

Crisis on the playing-fields

British secondary schools in the public sector are in a mess again. So they are elsewhere. But, in Britain, the chances of a cure are only poor.

THE playing-fields of Eton are no longer places where young gentlemen practise arcane games, such as cricket, which lesser breeds cannot understand. How could they be, when this year's adult English cricket team is suffering such humiliation in the Caribbean, and when most Eton students finish up as bankers and accountants? So much is now generally understood in Britain, where it is acknowledged that the great private secondary schools are among the few remaining citadels of the free-market meritocracy in intellectual life: the better schools will take in only the promising students, whose parents are given the opportunity of buying for their sons and daughters a superb education as modern as students' inclinations may dictate. So much, it may cynically be told, has always been the case, even when playing cricket well earned full social marks. The difference, now, not merely in Britain but in many parts of the United States, is that the educational privileges of the well-educated have become too narrowly concentrated even for economic comfort. Governments are coming to appreciate that modern states need larger numbers of skilled adults than the pure market mechanism can provide. So, of course, they should.

On present form, the chances that these fond wishes will be satisfied are, unhappily, only meagre. So much should now be clear from the extraordinary occasion last week when Sir Keith Joseph, the British Secretary of State for Education and Science talked for an hour to the largest of the British teachers' labour unions in utter silence. The teachers were no doubt forewarned that response of any kind, even hostile, might be read as an acknowledgement that the minister has a case. After a year-long dispute about pay from which nobody has benefited and from which students in the publicly maintained schools have suffered grievously, and which is still not settled, this trappist comment on official policy is understandable. What should be worrying not just the British government, its teachers and their students but also the young people whose fear of unemployment is not yet matched by a sense of how education might be a defence therefrom is that the impasse in the government's management of its schools promises that there can only be worse to come.

Sadly, all this has come about under a minister who is trying his best to say the right things. There is much in the view that in the past quarter of a century, British public secondary schools have been put through a series of enforced (in the sense of externally dictated) changes to which they have been entirely unable to accommodate. The well-intentioned movement to reform the curriculum in the 1960s is seen, in retrospect, to have been predicated on the assumptions that financial resources would always be generously available, that all teachers would always be more dedicated to their students' needs than to their own and that there would be more hours in the teaching week than there are in the adult working week. This revolution might have been accommodated by the schools if it had not coincided with the weakening of the schools as institutions in relation to the strengthened local education authorities of the same period, often staffed by failed teachers, and by the pressure first to reorganize schools to make their promise of equal opportunity explicit and, second, to do this in such a way as to pretend that equality among students is a fact. The present minister and his

predecessors in the present government may claim that they have been attempting only the remedy of the mistakes of previous administrations. But they have failed.

Understanding what has gone wrong must be a prelude to any successful course of action. Too much energy has been spent on ridding the secondary education system of the symptoms of malaise. At the outset, from a proper and justifiable concern with educational standards, the government resisted plans for liberalizing school leaving examinations so as to dilute the characteristic specialization of the English school curriculum. Repentance has come late, perhaps too late. Similarly, having seen the obvious gulf between the quality of the education to be had in private and public schools, the government invested (modestly, it must be acknowledged) in a scheme for allowing less well-off students to attend private schools when its efforts might have been spent on the improvement of the state sector. More recently, the government has sought to expose individual secondary schools to a simulation of the forces working in the private educational market by giving parents the right to choose which public schools their children would attend. The calculation was that the better schools would prosper and that the others would languish; the reality, when school rolls have been falling and populations have been shifting, has more often been to ensure that goodish schools have shrunk for reasons that are strictly spurious, and that their teachers have been demoralized as a result. Yet the hunt for a more "effective" way of simulating the market apparently continues, when the most urgent need is that schools of all kinds should be helped to recreate the sense of institutional identity they have been forced to abandon.

A part of Sir Keith Joseph's package of reforms might succeed in doing just that if the teachers were not now so alienated. Having rightly discovered that a government cannot complain about the quality of secondary schools if it delegates their management entirely to groups of locally elected politicians and their salaried officials, the British government seeks to play a more centralized role. That is fair, indeed inescapable. It also seeks to make schools into more nearly autonomous units, with budgets in whose spending there would be a large measure of delegation, and with policies and curricula distinctively moulded by those who teach in them. That too makes sense. The wayward element is that of parental choice, the notion that decisions made by students and their parents should determine which schools prosper and decline. Nobody will dispute that schools should serve the needs of the communities that look to them for education or that there should be mechanisms by which teachers, parents and students can work out a mutually acceptable policy. But to suppose that decisions about the quality of teaching at particular schools are too difficult to be made by adult educationists but that students can make them accurately when choosing between one school and another is an offence against reason.

Sir Keith Joseph may fairly complain that he has had no help from the teachers' unions in the evaluation of their own performance, or of their schools. Even if his desire not to let public education become a bottomless pit for public money had not led him to pay important sections of the teaching profession (such as



that which teaches science in secondary schools) so little that they are no longer renewing themselves, that might still have been the case — but at least it would then have been possible to tell where the fault now lies. The danger ahead is even more serious, for there is now every sign that British public education will be made an issue at the next general election some time in the next two years. If what the schools need is some peace and quiet to evolve an educational mechanism for fulfilling their obvious social function, another bout of quarrelling will have the worst possible effect. But if peace and quiet seem unattainable, there may be no choice. □

Oil keeps falling

We should worry about some of the casualties of cheaper oil.

THE price of oil has fallen further and faster than anybody had reason to expect. With the price of a barrel of crude oil hovering above or below \$10, those concerned are alternatively cheerful and depressed. In most industrialized countries, the outstanding prospect is that inflation will be further reduced, marked by the further prolongation of the long bull market that the stock exchanges have seen in the past few weeks. Among the unexpected benefits of just this development is that the US Congress, already locked into its attempt to move towards a balanced budget, will have to struggle a little less hard than it had feared.

The other side of that coin is comparatively inconspicuous as yet. It is too soon to declare the state of Texas a disaster area even if Phillips Petroleum is only the latest oil company to announce a reduction of exploration for new oil (by 36 per cent). And for the time being, the threat that municipal bonds sold by communities in Texas and Oklahoma may have to be rated along with less than copper-bottomed investments is probably less immediate than it seems; Texas, at least, may benefit a little if the word gets about that is a little more like other parts of the world. And there are no tears to shed for those oil companies, and the traders who dance attendance on them, that complain they have had their fingers burned by holding stocks bought more expensively; the roundabouts will be back before too long.

The much more serious casualties of these downs of the oil market are developing countries which depend on oil for their export earnings and which have, in the past 15 years, saddled themselves with a load of debt in the belief that they could thereby shake off their dependence for good. Mexico, with foreign debts on close of \$100,000 million, is the first in this despondent line. Venezuela and Indonesia are hardly in better shape. What will happen to Iran and Iraq it is hard to tell, except that their mutual war will now be less sustainable. Nigeria has been in trouble for many months already.

All these countries have in common not merely their now-suspended ambition to finance development from the revenue from selling oil but the circumstances that their revenues will fall immediately, while the benefits that may accrue from other developments, reduced interest rates or the increased exports of other kinds that may follow a growth of world trade, will by comparison be long delayed. They are also countries in which the process of development has not begun to moderate the plight of the poorest sections of their communities. In the industrialized world, there may now be a temptation to say that countries such as Nigeria have only themselves to blame for once having belonged to the Organisation of Petroleum Exporting Countries, or that those like Mexico would not now be so much in debt if they had been less corrupt in recent years. But this will not excuse the West, which now calls the shots, from dealing sympathetically and constructively with the problems that have been thrown up. Briefly, they all need more time to pay their debts. It is not a matter of charity but of self-interest. For just as the price of oil has fallen in response to market forces, it will be forced up again by the same forces. □

Visas back in favour

It is good news that the US and Soviet academies are to exchange people again.

THE decision of the Soviet and US academies that the time has come to breathe new life into their programme of scientific exchange which has been defunct for the past few years is a step in the right direction. Much good may come of the new development, especially if all those concerned are conscious of the difficulties that have previously brought similar programmes into disrepute. That is one of the reasons why it may be constructive, even though the US-Soviet agreement has been revived, that the old difficulties should be disinterred.

The first thing to say about the new agreement, as about the old, is that its formally specified quota of visits in each direction amounts to very little. Fifty man-months a year in each direction would be easily used up by the attachment of a few graduate students from one country to an institution in the other. The academic traffic that would be generated between countries such as the Soviet Union and the United States if universities were free to invite interesting people from elsewhere, and if those invited were then free to accept, would far outstrip the provisions now laid down. One of the potentially beneficial features of the new arrangements is that it will no longer be necessary that informal visits of this kind should be counted against the formal quota, but it should be widely understood that the past interchange of people between the two countries, even in good times, is meagre compared with the scale on which professional colleagues would be rubbing shoulders in normal circumstances. In short, there is a long way to go to normality.

The reasons why past experience of the operation of exchange agreements has been disappointing are easily catalogued. First, there is much less demand from individual scientists to travel for an extended period from the West to the Soviet Union. Language is a large part of the difficulty, but so too is the inevitable fear that a period spent in relative isolation from colleagues and competitors may punch an awkward hole in a career. Even so, it is remarkable that so many people in laboratories in the West have spent some time in a Soviet laboratory.

These are the pedestrian impediments to free exchange. The contentious issues of principle that have come up over the years are necessarily more difficult, and will never entirely be exorcised. One perennial trouble in dealings between the Soviet academy and its opposite numbers elsewhere is that the people who may be invited to, say, international meetings are not invariably those who eventually appear. However ridiculous it may be that people who have made internationally acknowledged contributions to important fields of research may never have been able to tell their tale to an international audience, people in the West who wish to see the new agreements succeed must not be tempted to use them as a way of teasing the Soviet system by means of repeated invitations to the same cast of characters who are not allowed to travel. Under the terms of the new agreement, this difficulty will be formally avoided by the provisions that each academy will nominate its own travellers, who may then be vetted by the recipient institution, but that will not (and should not) entirely banish hopes that exchanges will soon be much more free. The best route towards free exchange is constructive experience at the modest level now foreseen.

There are also more comprehensive political difficulties that are certain to arise. In the United States, for example, there will be one strong body of opinion that, while the Soviet Union refuses exit visas to those who wish to leave (for, say, Israel), no agreement can be worthwhile (or even consistent with the Helsinki accords). There is much in that view, but this is not the time to press it hard. Similarly, there will be many in the United States who fear that more visitors will mean more secrets lost, but that too is both an illusion and an irrelevance which should be buried for the greater good, at least for the time being. □

Genetic engineering

USDA goes too public too quickly

Washington

THE US Department of Agriculture (USDA) appeared to have shot itself in the foot last week when it disclosed that its licensing division approved for sale on 16 January the world's first genetically engineered live virus vaccine without informing its own Agricultural Recombinant DNA Research Committee (ARRC). Senior researchers at the department who sit on ARRC were fuming that they found out only at second hand.

The vaccine, developed and sold by Biologics Corporation of Omaha, Nebraska, prevents pseudorabies, which is caused by a herpes virus that primarily affects pigs, killing young animals. Antibody-positive animals have to be slaughtered, and an infected herd can transmit the virus to cows and sheep, in which it is fatal.

USDA is trying to eradicate the disease in the United States by a vaccination programme. One was vaccine, sold as "Omnivac", employs an engineered Bucharest strain of pseudorabies virus, patented by Dr Saul Kit and Dr Malon Kit of Novogene Inc. The engineered virus lacks the thymidine kinase gene apparently responsible for the pathogenic effect of the wild-type virus and which allows the virus to "hide" in latent form in the central nervous system.

Dr George Shibley, of USDA's biologics licensing division, says that the product had been approved after extensive field tests last year had established the safety and efficacy of the vaccine to the division's satisfaction. Division head David Espereth said proper procedures had been followed and that formal review by ARRC "would not have added anything" because the genetic engineering had involved only a deletion, not the introduction of a foreign gene. The division had established that host range and virulence were unchanged, but did not consider use of a vaccine to constitute an environmental release and so believed that no formal assessment of environmental consequences was necessary. Shibley pointed out that other artificially selected pseudorabies live vaccines are already on the market, and that the division has previously approved inactivated genetically engineered viral vaccines.

Shibley says the application was mentioned informally "two or three times" at ARRC meetings. But others on the committee deny hearing anything about it and consider that a formal notification should have been made. Another proposal to re-

lease genetically engineered organisms (tobacco plants) made by Calgene has been under review by ARRC for a year. John Fulkerson of USDA's research division believes that, whether or not formal procedures were violated, the Biologics Corporation proposal should have been fully discussed in the department and by others if necessary. Shibley concedes that, with hindsight, he would have raised it formally in ARRC.

The controversy has been music to the ears of opponents of genetic engineering and will do little to reassure an apprehensive public that biotechnology is under adequate control; Jeremy Rifkin of the

Foundation on Economic Trends claimed on the front page of the *New York Times* that USDA violated federal guidelines. And less than three weeks ago a California biotechnology company, Advanced Genetic Sciences, was fined \$20,000 and had its permit to conduct field tests of a genetically engineered microorganism suspended for falsifying test data.

Independently of the Omnivac rumpus, the General Accounting Office (GAO) last week released a report critical of USDA's biotechnology regulatory activities. In particular, it found that ARRC had neither the authority nor the direction to act effectively now that the Recombinant DNA Advisory Committee of the National Institutes of Health has agreed to assume a lesser role in regulation of non-biomedical biotechnology. USDA, with other government agencies, will shortly be publishing revised policy guidelines for regulating recombinant DNA products and research.

Tim Beardsley

SDI

Senators' scepticism fortified

Washington

OF the controversy still raging around the Strategic Defense Initiative (SDI) programme of research into ballistic missile defences, the most recent and scathing is a study of SDI carried out for three US senators and made public last week. It concludes that there have been "no major breakthroughs" to make deployment of a ballistic missile defence more feasible since President Reagan announced SDI three years ago.

The study warns that the current SDI research programme, which requires a decision on development of a missile defence system to be made in the early 1990s, could compromise promising long-term research by imposing an arbitrary schedule. And, in its effort to maintain public support, the study says, SDI may degenerate into what one senior scientist calls "a series of sleazy stunts".

The claim that there have been no major breakthroughs contradicts public statements by several administration officials. Former White House science adviser George Keyworth, for example, has said that the United States will be able to demonstrate technical feasibility of a laser-based defence in the early 1990s. Secretary of Defense Caspar Weinberger has spoken of barriers to progress "crumbling".

The Senate study, based on interviews with SDI researchers, was conducted by staff of Senators Proxmire, Johnstone and Chiles, Democrats on the Senate defence subcommittee who have been critical of what they see as excessive spending on SDI. Although not a comprehensive technical assessment, the study (which is

seen by the Pentagon as unduly pessimistic) will do nothing to change the senators' views.

The study acknowledges that several SDI projects have yielded results. But it says there are still "myriad uncertainties" about the programme as a whole, and that, on key points, the difficulties have become more serious than previously recognized. The study reports that SDI researchers at Sandia National Laboratory have concluded that space-based boost phase defences — which many consider an essential component of a credible anti-ballistic system — can never be made survivable, unless by treaty.

The study warns Congress to be critical about priority shifts made in SDI in an effort to keep on schedule, and points to contract overruns that have already obliged the Pentagon's SDI organization to settle for second-best components. The study urges Congress to delay a decision on whether to proceed with development beyond the early 1990s.

Whatever the technical problems, SDI's political battles are far from over. Although there is a strong consensus in Congress in favour of research into missile defences, how a candidate system would be tested without violating the 1972 anti-ballistic missile treaty remains unclear. The Pentagon has re-evaluated the treaty and concluded that it does not after all forbid testing in space — contrary to previous interpretations and the views of some other government agencies. Pressure to abandon the restrictive interpretation can be expected to increase as possible SDI weapons demonstrations approach the testing stage.

Tim Beardsley

French science

New government decides slowly

THE future of French research and technology policy-making was still in doubt last week, after another meeting of the government's cabinet, the Conseil des Ministres, which failed to mark out a division of responsibilities in this area.

At stake is the control of bodies such as the Commissariat à l'Energie Atomique (CEA), which controls nuclear power and atomic weapons research and much else besides, and of programmes such as the previous government's efforts on behalf of French electronics and computing, control of which has been wrested from the French telecommunications agency, PTT, but so far has passed to no other body. There are doubts too about just who will have responsibility for the medical research council INSERM, the agricultural research council INRA and many other smaller bodies.

What is going on is a kind of asset-stripping from the previous Ministère de la Recherche et de la Technologie (MRT) which, amid much resistance, had garnered control in 1981 of all the major French research bodies, or at least those concerned with civil research. This allowed centralized research planning, but left officials and some ministers (at the ministry of health, for example) with less control over research than they would have liked.

Now that process appears to be being put into reverse, but the slowness with which decisions are being made — some close to the process say it will be a month before much more is known — indicates there may be some conflict within the new government about how power is to be divided. It is even being suggested that Alain Devaquet, the new minister for higher education and research, is beginning to see some of the advantages in MRT's power, and may be unwilling to accept the idea of too great a loss of control, or of the establishment of a prime-ministerial body above him with the overall job of research coordination (as was the practice in previous right-wing governments). "But the longer the delay, the more Devaquet's power is likely to slip away", said one seasoned Paris commentator.

Meanwhile, the future of one of the most important institutions established by the previous government is also under a cloud. The "evaluation bureau", provided by the previous science minister's three-year plan but accepted by the French parliament only last December, was to have carried out independent evaluations of the work of all the major French research institutions, from CEA and the Centre Nationale de la Recherche Scientifique (CNRS) downwards.

Little more than a week before the elec-

tions, M. Hubert Curien, then minister of research and technology, put the deputy-director of his advisory cabinet, M. Jean-Pierre Chevillot, in charge of this evaluation system. He was to establish evaluation committees and procedures, and set in motion a review — initially — of the finances and policies of CEA, CNRS, INRA, INRIA (for informatics) and

Argonne National Laboratory

US keeps fast reactors alive

Argonne, Illinois

NUCLEAR power plant operators typically do not welcome visitors when the safety systems are disabled and primary cooling systems are shut down. But engineers from Argonne National Laboratory (ANL) made sure they had a crowd of onlookers in ringside seats at their Idaho Falls test facility last week when they purposely simulated just those conditions in their Experimental Breeder Reactor (EBR-II).

"It was truly an impressive performance", says Chuck Till, assistant director for engineering systems at Argonne. "Within 100 seconds after the pumps were shut off, we came down to zero power."

Because EBR-II is pool-type reactor, the core is surrounded by a lake of liquid sodium with high thermal inertia. When coolant flow around the core stopped, the core began to heat up rapidly. But long before it reached the sodium boiling point, the metal fuel pins and control rods expanded, passively terminating the chain reaction.

It worked on the blackboard, it worked in computer simulations, and the engineers present were willing to bet their lives that it would work in practice. But when a pressure valve in a heat sink line popped open with a loud bang, everyone in the control room jumped into the air.

The successful test of EBR-II sets the stage for further development of the Integral Fast Reactor (IFR), a project Argonne hopes will play an important role in the future development of nuclear power.

The decision to proceed with IFR was bold. The death of the Clinch River Breeder Reactor in Tennessee threw the US nuclear programmes "into chaos", says ANL director Alan Schriesheim. But it also opened "a window to change the course of reactor design", says Yoon Chang, general manager of the IFR programme. Adds Schriesheim, "one page in our energy portfolio should be nuclear".

Pool type reactors are not new; several are in operation around the world. But using metal fuel represents a major

ANVAR (for research support in small companies).

These studies and other like them were to result in published reports and long-term policy review which could have been, and could yet be, an effective means of countering a strong French tendency to institutional sclerosis and atrophy of the mechanism for accountability and feedback. But will the project continue? "I've put the question to the minister", said Chevillot last week. He is still awaiting a reply.

Robert Walgate

change from current technology. Till believes that IFR will prove the advantages of the metal fuel design, and may then provide the incentive for a shift away from current oxide fuel technology, despite huge investments in that process.

As the flagship of ANL's nuclear programme, Schriesheim is pleased at the success of IFR, which is just one part of the "strategic plan" developed by ANL to keep the laboratory operating and moving in a direction consistent with national research priorities.

In the past decade, ANL has been on a roller coaster. During the "energy crisis" of the 1970s, it was overflowing with money, personnel and demonstration projects. In the Carter era, says Schriesheim, money was "shovelled" at energy programmes, and ANL's staff grew from 4,000 to 5,700. The Reagan administration, however, brought massive cuts in programmes of energy conservation, solar energy and alternative fuels; ANL's staff shrank to 4,300. Following Clinch River's demise, there were further cuts, bringing the number of personnel down to 3,700.

The strategic plan was an attempt to reverse that slide. "Laboratories make a mistake if they think they can sit back and money will roll in", says Schriesheim. In addition to strong support of a nuclear programme, ANL proposed major thrusts in new facilities, most notably the 6 GeV synchrotron light source.

While support from the Department of Energy has grown, Schriesheim has also sought support directly from Congress. ANL's proposed 1987 budget is below the \$260 million approved in 1986, but Schriesheim is hopeful that Congress will restore any cuts. Unfortunately, he now must fight for funds for alternative energy strategies at the very time that oil prices are plummeting.

"True, oil prices are falling", says Schriesheim with a rueful smile. "But in the future we'll have to go for alternatives." It is Schriesheim's hope that when the country looks to those alternatives, ANL will be there with the answers.

Joseph Palca

US – Soviet pact

Cooperation agreement signed

Washington

THE US National Academy of Sciences last week signed a two-year cooperation agreement with the Academy of Sciences of the USSR providing for exchange of research scientists, joint workshops and regular meetings of academy officers. Joint meetings were suspended in 1980 by the US side in protest against the internal exile of Soviet dissident Andrei Sakharov. The new agreement expands a protocol signed by the presidents of the two academies in January 1985.

A significant feature of the new agreement is that it specifies joint selection of scientists participating in workshops. This is intended to answer past criticism in the United States that the Soviets avoid sharing their best work by denying US access to their best researchers, especially in fields such as mathematics, where they may hold an edge over the United States. In addition, each academy agrees to support "insofar as possible" invitations issued to particular scientists by the other academy.

Individual exchanges have continued during the Sakharov protest at about 50 man (or woman) months in each direction

per year. The new agreement includes the same 50-month annual quota on exchanges in each direction for scientists nominated by the sending academy, but there is to be no upper limit on the number of visits by invitation.

The US National Academy of Sciences has been criticized by hardliners within the US administration for seeking to foster scientific collaboration when in their view exchanges increase opportunities for Soviet spying. Dr Frank Press, president of the US academy, said last week that such critics were "generalizing from a few bad examples". As a nod at concern about human rights, the new agreement specifies that all activities will be based on the Helsinki Accords. And, perhaps to forestall criticism from Sakharov sympathizers, the US academy this week sent a telegram to the president and members of the Soviet academy asking them to do all they could individually and collectively to ameliorate Sakharov's condition. The US academy also planned this week to release new details of its past activities in support of Sakharov.

Tim Beardsley

An ombudsman for laboratory animals?

THE world's first professor of animal welfare, Dr Donald Broom, is likely to be much in demand when he takes up his new post in September. The new chair, at the University of Cambridge Veterinary School, has been funded by the Animal Welfare Foundation, set up in 1983 by the British Veterinary Association. And despite the broad terms of reference attached to the post, the keen interest shown by the popular press in the activities of the more extreme animal liberationists should mean that he will be constantly in demand to put

the "academic" case for experimentation on animals.

Dr Broom's present post is reader in pure and applied zoology at the University of Reading, where he has specialized on studies of farm animal behaviour and welfare. He plans to continue work on zoo and farm animals, but may consider other areas — animals as pets or in experiments, for instance.

A main aim of the work is to find ways of telling just how an animal is reacting to its environment. This is a topical problem, since one of the most contentious parts of the bill on laboratory animals at present going through the British parliament is the "pain clause", discussion of which continuously founders on the problem of deciding how much discomfort an animal is experiencing. Dr Broom says that "if there is some good reason for keeping animals or interacting with them in any way then we have the responsibility... to modify our treatment of the animals so that their welfare is as good as possible".

Dr Broom's past achievements include proving the benefits of scaring pigeons rather than killing them, and finding more humane ways of transporting poultry. His next target, of accelerating the "constructive discussion" on animal welfare issues, is a daunting one; emotion has so far eclipsed rational discussion.

Charles Wenz

Marine uranium

Japan plumbs the depths

Tokyo

THERE are something like four thousand million tons of uranium dissolved in the sea: more than enough to run 1,000 nuclear power stations for 40,000 years. The problem, of course, is to find some way to extract it efficiently. There seems no easy solution but this week Japan's Metal Mining Agency takes the first step towards extracting at least a small part of this limitless treasure.

To be more precise, it hopes to obtain 10 kg of uranium within a year. This may not sound like much, but it is 1,000 times more than anyone else has so far succeeded in extracting in a year. It will come from the world's first large-scale sea-water extraction pilot plant which is just about to begin operation at Nio-cho in Kagawa Prefecture on the island of Shikoku.

The plant has been built by the Metal Mining Agency, an agency affiliated to the Ministry of International Trade and Industry's Natural Resources and Energy Agency at a cost of 3,300 million yen (US\$183 million). Only 0.003 parts per million of uranium are found in sea water and to bring the concentration up to usable levels, extraction takes place in two stages. First, 1,700 tons of sea water per hour are to be pumped through beds of particles containing specially developed titanium hydroxide absorbents. The concentration of uranium increases 7,000-fold. Next, the absorbed uranium is eluted with dilute hydrochloric acid and passed through an ion-exchange resin which increases concentration another 140-fold. The result, in laboratory tests, when the resin has been washed out, has been concentrations of uranium close to 3,000 p.p.m. — slightly better than natural uranium ore which contains 2,800 p.p.m. of U_3O_8 .

Research on the extraction of uranium from sea water is under way in both the United States and West Germany where an unofficial world record was claimed at Kiel University with the recovery over several years of a total of 150 grams. If the Japanese plant reaches its target, it will be a considerable advance. Beyond that, various plans are in the air. To run an extraction plant efficiently, it is necessary to develop suitable absorbents and to find ways to minimize the energy required to pump enormous volumes of water through the absorbent. One proposal involves the construction of a plant on a causeway across a strait with a strong tidal stream. Another possibility is an extraction ship which sucks up uranium as it ploughs through the waves.

Alun Anderson



Donald Broom

Dioxin exposure

CDC study still at square one

Washington

A \$60 million epidemiological study of the health consequences of exposure to contaminated defoliant by dioxin used during the Vietnam war has been suspended, and may be cancelled before it can ever get under way. Who will make the final decision to proceed, and even when the decision will be made, is still unclear.

Agent Orange was the code name for a defoliant agent used by US forces during the Vietnam war. One contaminant of Agent Orange is known to have been the dioxin isomer 2,3,7,8-TCDD, a known carcinogen in animals. But the health effects of dioxins in humans have been the subject of long and heated debates. Even studies of acute high-level exposures have given equivocal results, confirming that the possible health effects of long-term low-level exposure to toxic agents cannot easily be answered.

Although the skin disease chloracne is the only clear-cut health problem in humans, studies have implicated liver disease, soft-tissue sarcomas and birth defects in children of exposed adults. But an Air Force study of soldiers actually involved with Agent Orange spraying, termed the Ranch Hand study, failed to uncover health problems unequivocally associated with the defoliant.

Still, anecdotal evidence for the effects of Agent Orange from Vietnam was strong. Under pressure from veterans' groups, Congress in 1979 declared that a study should be done to provide definitive answers. The Veterans Administration was the natural first choice for the agency

to carry out the study. But those concerned about conflicts of interest argued that the study should be moved to the control of the Centers for Disease Control (CDC) at Atlanta, Georgia.

Initially, CDC were alarmed that it might be impossible to find a large enough cohort of soldiers who had not been exposed to Agent Orange. As things worked out, just the opposite was the case. Records of troop movements failed to yield easily interpretable data for assessing the exposure of individual soldiers.

Carl Keller, chairman of the science panel of the Agent Orange Working Group (AOWG), says the difficulties are "horrendous". AOWG is the executive branch group charged with looking into the long-term health effects of herbicides. The Under Secretary of the Department of Health and Human Services (HHS) chairs AOWG, and ultimate responsibility for seeing that the Agent Orange study is "scientifically valid" falls to him. Donald Newman, who joined HHS last year is still awaiting Senate confirmation as under secretary.

Meanwhile, Congress charged the Office of Technology Assessment (OTA) with the assessment of CDC's protocols. By law, no study can proceed without OTA's blessing. CDC's proposed study had originally consisted of three groups of 6,000 subjects each; a high-exposure group, an unexposed combat control group and a non-combat unexposed group. But finding combat controls proved easier than expected, so last November, CDC presented OTA with a new

protocol, calling for only two cohorts of 6,000 subjects each. CDC planned to start interviewing prospective study participants in January this year, with extensive physical examinations to follow. But OTA balked, saying that CDC had failed to establish adequate exposure criteria and did not address problems associated with mis-classification of an individual's exposure history. At OTA's suggestion CDC agreed to postpone further activity until these issues could be resolved.

AOWG's efforts to help CDC settle the exposure issues seems to have backfired. AOWG formed a subpanel, chaired by Alvin Young of the White House Office of Science and Technology Policy, to review possible solutions, but the selection of Young raised concern that the momentum of the Agent Orange study might be waning. Young, who is described as having grave doubts that any workable exposure measurements can be found, declines to comment on the deliberations of his panel.

Finding an acceptable exposure measure is crucial to the CDC study, but nobody thinks that will be easy. Peter Layde, acting director of the Agent Orange programme at CDC, says that there is no obvious reason why one measure should be more meaningful than another, and that the many exposure estimates used so far have so much variability that they are meaningless.

For veterans' groups, the delay has been frustrating. "Confusion over data on exposure should have been resolved a long time ago", says John Terzano of the Vietnam Veterans of America, who is anxious to have a study that people will listen to even if they do not agree with it.

Some new information on the health effects of Agent Orange exposure will come from other studies under way at CDC. The Vietnam Experience study compares 2,000 in one group of people who served in Vietnam with another 2,000 whose military service was elsewhere. A case control study of soft-tissue sarcomas will shed some light on possible links between Agent Orange exposure and cancer.

CDC would like to begin physical examinations of participants in the Agent Orange study in June, when the Lovelace Clinic in New Mexico will be finished with the Vietnam Experience Study examinations. But after Young's group makes its recommendations, AOWG must approve them and transmit them to CDC. CDC must then change to a new protocol, and OTA must then evaluate it. For all that to happen by June needs unusually quick action.

Keller concedes that AOWG or CDC may decide it is not possible to quantify indirect exposures, so no meaningful study can be undertaken. But if that is the decision, warns Terzano, "a very, very serious problem is brewing". **Joseph Palca**

Hewlett thanks Stanford University

Washington

In one of the more generous gestures of alumni appreciation, William Hewlett last week gave Stanford University \$50 million, the largest individual commitment the California institution has received in its 100-year history. The gift from the co-founder of the Hewlett-Packard company will help to revive Stanford's sagging research facilities and increase financial awards for the university's 12,000 students.

Hewlett's pledge will also be used to mark the inauguration of his university's centennial campaign, which formally begins in 1987. Hewlett and Stanford's president, Donald Kennedy, have been discussing its dispensation for at least two years, with some clearly defined results. All but \$10 million of Hewlett's gift will be ploughed into the 41-acre Near West campus, between the main campus and Stanford's medical research facilities.

Although space attractions have yet to be worked out, university planners expect to tear out 300,000 square feet of the campus's 725,000 total building area, and to add 800,000 square feet of floor space specifically designed for the new equipment included in renovation blueprints.

Stanford's research rejuvenation will cost \$250 million in total, and will be completed within a decade. The university will use the remaining \$10 million of Hewlett's pledge to establish endowed undergraduate scholarships and graduate fellowships.

William Hewlett has a history of beneficence to Stanford, whose endowment already nears \$2,000 million. But the 1934 graduate may have good reasons for his gratitude: it was at Stanford that Hewlett first met David Packard and forged the friendship that has grown into a billion-dollar business whose headquarters remains a few miles away. **Karen Wright**

Spanish science

New law in effect at last

from our special correspondent

Barcelona

THE Spanish parliament has at last approved the science law which the government has been preparing for several months. But even now, it is far from clear what the effects of the new framework will be, although the fact that the law, called the Ley de Fomento y Coordinación General de la Investigación Científica y Técnica, is the first to have brought research within a general framework cannot but be significant.

The new framework will include all major research institutes in Spain. No fewer than ten ministries of the government will be involved in the operation of the law, together with representatives of several autonomous regions. While the law itself does not define research as a national priority, one of the first of the tasks required within the new framework will be the preparation of a national plan for scientific research and technical development.

As part of the new development, it has also been agreed that the budget of the principal organization for the support of scientific research, the Comisión Asesora de Investigación Científica y Técnica (CAICYT) will probably be increased in the current year by about 36 per cent above that for 1985, when CAICYT had 9,112 million pesetas (roughly \$60 million) to spend.

CAICYT at present spends money on research grants in universities and by means of joint ventures with industrial companies and other organizations. The trend begun by the centrist government of 1981–82 of designating certain areas of research for special attention, and which has been reinforced by the present socialist government, is likely to be continued, with special attention being paid to research in biotechnology, aquaculture, agroenergetics, microelectronics and high-energy physics, which last year accounted for nearly a quarter of total spending.

In general, however, the new law is permissive only, which means that the amounts of money available in future years, and the numbers of posts that will be created within the new framework, are at present unspecified. Moreover, the composition of the key advisory committee, the Consejo Asesor, is for the time being undefined, although the government says that the composition of the committee will be decided in the next three months. Much will depend on the balance in the membership of the new council between working scientists, industrialists, administrators and government officials. But the provision for better co-ordination between the ministries of in-

dustry and of education and science is welcome, while much is expected from the improved interaction between the public and private sectors.

General reactions towards the new law so far are mixed. The measure has been criticized by some for its neglect of questions such as the proper relationship between the central and the regional governments. Professor A. Nieto, a former president of the Spanish research council (CSIC) has for example pointed out that the Catalan legislation that gives the regional government "exclusive competence" within its territory is potentially a source of confusion. F. Mayor, previously a minister of education and science in the centrist government of the early 1980s, would have preferred that the evolution of Spanish science should be more closely determined by the creative energies of

scientists than by planning, to which the new law will give new encouragement.

Others point out that there is nothing in the new law to encourage the application of the process of peer-review not merely to the assessment of research grants but to the evaluation of the work of university departments and research institutes, where the influence of foreign scientists is still conspicuous by its absence.

On balance, the new law is a step in the right direction, although the development of science in Spain will be more directly determined by the practical decisions taken by the government about the budget for research and the appointment of people to the key posts within the new administrative framework. Certainly there is nothing in the new law to guarantee that the social status of Spanish science will in future be enhanced, nor is there an audible echo of the positive commitment to science by President Francois Mitterrand at the outset of his socialist government of France in 1981. □

China

Educational reforms ahead

CHINA has published a census of its civilian science and technology sector, the first of its kind since 1949. It shows that the country has 4,690 civilian research institutes, employing more than 770,000 people, including 231,000 scientists and engineers and 121,000 technicians. There are 760 universities and colleges of science, technology, agriculture and medicine, with a "technical work-force" of 481,088, including 356,088 scientists and engineers. Both the institutes and the universities and colleges are deeply involved in work for industry: in 1985, the institutes earned 780 million yuan by developing technology for industrial enterprises, while the universities signed 7,077 contracts with industry worth a further 126.4 million yuan.

Nevertheless, official approval of the links between research and industry is by no means unqualified. Speeches at a meeting of the new State Education Commission last month suggested that the relationship between the study of basic science and its application in the development of technology is not being "handled correctly". To produce a "rational structure of personnel", it was urged, higher schools should coordinate their efforts to avoid duplication. Schools of engineering, therefore, should concentrate in applying science to technological development, and colleges of science should concentrate on basic research "while also engaging in the development of technology". For those unable to study full-time, the planned television open university should enrol not only people already in a job but also middle-school leavers.

Educational reforms scheduled for this year, it was indicated, will be aimed at eliminating the egalitarianism persisting from the period of the cultural revolution ("everyone eating from the same big pot"), to which the current leadership is resolutely opposed. A pilot scheme involving a few universities and colleges will be launched this year by which student grants will be replaced by a system of scholarships to "harness the initiative of students and their conscientiousness in study, and to reward those who are both academically promising and morally outstanding".

A new system for doctoral dissertations and the conferment of doctorates in various fields including clinical medicine and engineering is to be worked out. Now that many colleges and universities have established postgraduate courses, postgraduate training should be concentrated mainly in China itself, the commission urged, and only "units in need of specialized personnel" should send students abroad. Foreign study tours by trained personnel will however continue to be encouraged as part of the policy of opening China up to the outside world.

An interesting feature of China's recent policy on foreign study is the practice of allowing certain people to study abroad at their own expense. This policy will also continue but it was urged that such people should be treated in the same way as those who study abroad at the government's expense and that they should be offered "correct guidance" to overcome the "problems and defects" that now exist among them.

Vera Rich

UK research

MRC units close

A SMALL decision by the Medical Research Council (MRC) at the end of March is being seen as a larger blow to the standing of British research in an area where it cannot afford to decline.

The decision is the closure of the MRC Mammalian Genome Unit in Edinburgh, following a year-long search to find a director to replace Ed Southern, who left to take up the chair of biochemistry at the University of Oxford last October. In line with its longstanding policy, MRC first assessed the desirability of maintaining the unit. The decision was strongly in favour of keeping it going but MRC failed to attract a new director who satisfied the criteria for the job.

Commenting on the failure this week, Sir James Gowans, MRC's secretary, said that the council had searched hard for a new director of sufficient calibre and that he had no idea why the search had failed. Perhaps the best people can command greater rewards than a small MRC unit can offer, he speculated. In any case, MRC would certainly continue to support the two key researchers in the unit.

The closure of the unit is being seen in some circles as a further worrying sign of the decline in standing of British research. If nobody can be found to fill such a post, it is argued, either Britain is not producing scientists of the necessary calibre or there are insufficient attractions to keep the best scientists in the country. Professor Southern, who has given his name to the key molecular biological technique of Southern blotting, said last week that whatever the reason for the failure to replace him, he was very worried about its impact on young scientists thinking of pursuing a career in Britain at a time when there is already difficulty in finding good postdoctoral researchers.

A second MRC unit that is to be closed apparently for lack of a suitable director is the Mineral Metabolism Unit in Leeds. Professor B.E.C. Nordin retired as director of this unit in 1981 and Dr Munro Peacock has been holding the fort during the search for a permanent successor. After a very long and extensive search it has been the unanimous view of a committee that included international experts that no suitable director could be found to take over, said Sir James. There is, however, a very strong need to maintain some of the research which concentrates on calcium metabolism.

Two other MRC establishments that seem destined to close are the Unit on Neural Mechanisms of Behaviour and the Developmental Neurobiology Unit. It seems that MRC does not intend to maintain these units after the forthcoming retirement of the directors. **Peter Newmark**

It's raining pesticides in Hokkaido

Tokyo

JAPAN may be the first country to be suffering from "pesticide rain". There is mounting evidence that pollutants found in Lake Mashu, in the northern island of Hokkaido, have come all the way from the Chinese mainland before being washed out of the sky.

Lake Mashu is a 7,000-year-old lake created when the summit of a volcano collapsed, forming a caldera which filled with water. High cliffs rim the lake and prevent any streams from flowing directly into it: all the lake's water comes from rain or from water draining through the rock. Human influence has been kept to a minimum and visitors are not allowed down to the edge of the lake itself. The result is a lake of exceptional clarity — it is often claimed to be the clearest in the world. In 1979 it was possible to see down to a depth of 117 feet (35.8 m).

The Environment Agency has been analysing the water of Lake Mashu periodically for the past four years and has discovered that it contains an increasing quantity of the pesticide benzene hexachloride (BHC). The pesticide, originally developed by ICI in the United Kingdom

and marketed as "Gammexane", has not been used in Japan for fifteen years because of suspicions that it was carcinogenic. It is still widely used in Europe and the United States, as well as in mainland China and Korea. Concentrations of BHC in the lake now average 32 nanograms per litre; still low, but in the summer the concentration in water at the surface becomes many hundreds of times higher. The rain falling at that time contains as much as 128 nanograms per litre, more than twice the levels detected 15 years ago when BHC was still in use in Japan. The only plausible source for the pesticide is China and Korea where BHC is often used as an agricultural spray: that means BHC has been transported more than 1,500 kilometres across the Sea of Japan before falling on Hokkaido.

BHC levels in the lake will probably continue to rise for the lake has no outlets and the pesticide is virtually non-biodegradable. It is still a long way from approaching harmful levels but that has not stopped there being some hard feelings about the way other nations can pollute one of Japan's most protected lakes.

Alun Anderson

US radioastronomy

Telescope bites cosmic dust

Washington

BARRING an eleventh-hour reprieve, the Clark Lake Radio Observatory (CLRO) in Borrego Springs, California, will close its doors on 26 April, shutting down a unique telescope that is just beginning to explore the low-frequency radio sky. A final effort to obtain funds from the National Science Foundation (NSF) fell through at the end of March.

CLRO operates in the range of 15–125 MHz, "unfashionably" low for much of radioastronomy, but with a dedicated group of enthusiastic users. CLRO played a critical part in the discovery of the millisecond pulsar; the characteristically steep spectra of such pulsars are conveniently detectable at low frequencies. The phased array at CLRO is also much more sensitive than other low-frequency instruments. CLRO recently completed a catalogue of low-frequency radio sources, increasing their number to nearly 35,000 from the 350 previously identified.

CLRO also provides the only two-dimensional imaging of the solar corona, according to Mukul Kundu, a professor of astronomy at the University of Maryland and one of the observatory's directors. The telescope can yield images of emission patterns at a rate of one every 10 milliseconds, and so can allow observation of rapid phenomena in the corona.

The University of Maryland owns and

operates CLRO but operating funds have come primarily from NSF. When NSF "pulled the plug", the university was loth to provide the minimum \$200,000 per year needed to keep the facility going; it did not help that the observatory was 3,000 miles away.

With a lot of prodding, the university agreed to provide just under half that sum if NSF would make up the difference. But when NSF declined at the end of March, Maryland gave notice to the four full-time employees at CLRO.

Bad timing has played a part in CLRO's demise. Three years ago, when NSF was evaluating CLRO's status, the telescope was still being calibrated. Only after NSF took the decision to scale down support did exciting results begin to emerge. Moreover, because of its small size, CLRO lacked the political muscle to rally the astronomical community to its aid.

At least a two-month reprieve may come from the Office of Naval Research. Erickson has interested the US Navy in using CLRO to investigate ionospheric phenomena.

Spurred by this initial interest, Erickson is planning to "knock on doors in Washington" to sell to others the virtues of CLRO as a tool for ionosphere research. But without a long-term grant, CLRO faces a hand-to-mouth existence for the foreseeable future. **Joseph Palca**

US education

Science planners ask for more

Washington

AN urgent plea for more federal support for undergraduate science and engineering education has been made by a special task committee of the National Science Board, the policy-making arm of the National Science Foundation (NSF). The board has endorsed the report*, which states that NSF support for undergraduate education should be increased by \$100 million by 1989 from its current level of about \$11 million, and has asked NSF director Erich Bloch to prepare a plan of action.

The new report provides much-needed ammunition for Bassam Shakhshiri, head of NSF's off-and-on science and engineering education directorate. The directorate, which was abolished in 1981, has faced an uphill struggle since its re-establishment in 1983, competing for funds with NSF's research directorates. Its current level of support, \$87 million, is still way below the peak in the late 1960s; almost all its growth since the nadir in 1981 has been for pre-college education.

The task committee, chaired by Homer Neal of the State University of New York, concludes that, unless immediate action is taken, the United States "will not meet its current requirements for science and engineering personnel, much less respond to future challenges". It identifies three problems that require "attention of the highest priority". They are: laboratory instruction (which is "too frequently carried out in facilities that are obsolete and inadequate"); faculty members (where there are in some fields "serious shortages"); and courses and curricula ("frequently out of date, unimaginative and poorly organized"). Faculty shortages in engineering disciplines occasion "extraordinary levels of concern": 8.5 per cent of budgeted positions are vacant.

Obedient to the current administration's belief that state and local governments should bear primary responsibility for pre-graduate education, the task force is careful to emphasize the obligations of governments other than the federal. Going beyond its mandate to consider NSF's role, the committee also urges that states, academic institutions, private industry and other federal agencies should improve their efforts. But it also says that NSF should take "bold steps to establish itself in a position of leadership" in undergraduate education in mathematics, engineering and the sciences. It proposes a \$50 million budget increase in 1988, rising to the target of an extra \$100 million the following year.

One of the committee's chief concerns is the projected decline in the size of the 18–19 age group. The first effects may

already be apparent; between 1973 and 1983, the number of undergraduate science majors fell by 15 per cent. In addition, science courses are becoming less popular. Faculty members are ageing and have little incentive to keep up to date in teaching. In some institutions, laboratory instruction is being abandoned in introductory courses.

The task committee's report can be expected to receive a friendly reception on Capitol Hill, which has traditionally been sympathetic to education. The welcome

Soviet rivers

Conflict of interest in academy

SOVIET plans for diverting major rivers have caused a row within the Soviet Academy of Sciences. Academician Dmitrii Likhachev, interviewed on Soviet television, has accused the Soviet presidium of bias and the "violation of ethical norms" in holding a conference on the proposed diversion of the waters of the north-flowing rivers of Siberia without inviting representatives of the humanities who, in Likhachev's opinion, should have been there to "defend the interests of culture". Likhachev is a member of the departments of history and of literature and language of the Academy of Sciences.

By the time of Likhachev's broadcast, the issue of the river diversion had already become somewhat theoretical. After considerable opposition (see *Nature* 319, 88; 1986), a paragraph relating to the river diversion project was omitted from the version of the guidelines for the next five-year plan (1986–90), adopted by the 27th Party Congress last month.

In fact, many of the participants at the conference were also opposed to the scheme, holding that the diversion is now unnecessary because, contrary to the forecasts of the All-Union State Institute for Water Resources, the level of the Caspian Sea has ceased to decline. Speakers are also said to have quoted an opinion of the World Meteorological Organization that the current increased precipitation in the Volga basin will be maintained for 50 years.

Others agreed that the river diversion plans were based on forecasts of water usage that took no account of how recycling methods in industry, and the more rational use of water in agriculture, will gradually reduce water consumption. Writing in *Pravda* in mid-February, a group of five eminent economists, agronomists and geologists noted that by reducing waste and "justifiably cutting irrigation norms" in the arid south, no less than 60 km³ of water could be saved each year, while the introduction of water-

will be enhanced by the committee's emphasis on schemes that require local governments to provide matching support for federal dollars; a subcommittee of the House of Representatives has already moved to increase funding above the administration's proposed sum for 1987, although there remain many obstacles. But, as for all federal expenditures, the Gramm–Rudman deficit reduction act makes predicting congressional action difficult. Whether the pleas will fall on receptive ears in the White House is less certain.

Tim Beardsley

*Report of the NSB Task Committee on Undergraduate Science and Engineering Education to the National Science Board, 1986.

conserving farming techniques would make it possible to use up to 70 km³ of local water annually for crop-raising in the southern steppes. These estimates are "many times higher" than the proposed volume of water (about 6 km³ annually) to be redistributed by the diversion scheme.

Likhachev's complaint is not based on the unexpected natural recovery of the level of the Caspian, although he mentioned that the rise in the water level has already flooded a number of health resorts built too close to the shore of the supposedly shrinking sea. He concentrated instead on what he saw as the ethical issues. Not only were the representatives of the humanities excluded from the conference, but the title of the conference was unethical in referring to the "first stage" of the diversion project. Likhachev said that the "first stage" should not be discussed without knowing how many more stages there would be, especially when the first stage was to go ahead "without a vote".

Likhachev clearly has little faith in official assurances that no relics of mediaeval architecture will be inundated by the diversion. He demanded to know why the population of the Russian north, "which has lived there since the twelfth century", should have to lose its monuments to provide water for the south. He was also concerned that the third stage of the diversion plan would affect the Pechora, whereupon, he said, the fate of the Komi people, "one of the most interesting and cultured people of the Russian north", would "be sealed".

Conflicts between the humanities and the exact sciences do not often manifest themselves in the Soviet Union. Likhachev's attack on the ethics of the academy presidium, although consistent with the policy of open criticism encouraged by the new regime, could well call into question the role of the humanities in an academy that is increasingly focused on science in its narrower sense.

Vera Rich

New idea on South Africa

SIR—We are a group of South African academics, two of whom are citizens by birth. We feel a great deal of sympathy with those people abroad who are attempting to bring about change in this country through the application of boycotts. Experience has taught us that the most effective actions have involved trade, and nationalistic activities, particularly in the field of sport. There is, however, also a strong argument for academic boycotts, as many of the people with whom we come into daily contact are very much a part of the South African problem. Few of our universities are multiracial, and even those that practise a form of integration are certainly not blameless.

One of the problems with blanket boycotts, however, is that they generate a sense of martyrdom, giving credence to the defensive argument that the rest of the world is concentrating on our problems to avoid confronting their own. More unfortunately, they fail to take cognisance of the efforts of people like Professor Phillip Tobias, who have openly fought racist policies in this country ever since they have been in positions to do so.

We should like to propose an alternative to an all-out academic boycott which, by its positive nature, might help to alleviate the unfortunate situation in this country. When academics from South African institutions submit manuscripts to international journals, or apply for admission to conferences overseas, they should be required to sign a declaration to the following effect: (1) that they do not support an academic or social system that is based on inequality or discrimination, and (2) that they are in favour of science as a united and cooperative activity which is not divided by artificial barriers of race, sex or religion. In this way, they will be forced to show their true colours, so to speak, rather than being able to fall back on the excuse that the rest of the world is fighting apartheid with a system that is just as discriminatory. Perhaps, to minimize South African paranoia, this practice could be extended to include all participants who wish to be considered members of an international community of scientists.

To be effective, this action need not be particularly radical. Even so, there might well be repercussions for South African researchers who signed such a declaration, in that most funding for research stems directly from the government. We feel, however, that this is a sacrifice that people should be allowed to make; for, if conference participants after signing such a document were refused permission to attend (by having their passports confiscated, as has happened in the past), or were deprived of the financial support that

should reasonably be theirs, the world would be alerted to a clear case of academic persecution.

This would contribute to the already strong polarization among academics in this country. Scientists would be forced to decide whether to remain part of the international academic community, or to support the isolationist South African government. Many people are riding on the shirt-tails of our liberal institutions, without actively supporting an end to apartheid. We feel that the time has long passed for ambivalence with regard to the issues confronting this country. It is important for the academics who have a commitment to the future of a non-racist, democratic South Africa to unite against apartheid policies.

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The role of advocacy

SIR—N. Hetherington's commentary on legal eagle Edwin Hubble (*Nature* 319, 189; 1986) considers advocacy as an integral part of science, to be recognized and studied. I agree that the subject should be studied.

Scientists are normally concerned with collecting data, and publishing their findings in the literature. The published data provide a basis for other scientists to make independent judgements on the correctness or incorrectness of hypotheses. However, if a scientist "orchestrates" the evidence in such a manner that only selected data are published, the ability of peers in the scientific community to evaluate hypotheses may be seriously impeded. If "advocacy" in science means the selective presentation of available data for publication in the literature, I believe it is problematic.

Advocacy is an integral part of law. Attorneys are normally concerned with achieving a favourable result for the client. Advocating a theory or position that may achieve a favourable result for a client is not necessarily consistent with full revelation of the available facts.

Science involves a search for truth, rather than the achieving of favourable results for clients. Perhaps advocacy should be left to the legal profession, and scientists should concentrate on full, unqualified disclosure of all available data to their peers.

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More on Sakharov

SIR—I appreciate all that Zhores Medvedev¹ has said on the scientific merit of Andrei Sakharov. My letter² exhibits only my own understanding that a head of a scientific school is a person who promotes current research as leader of a group of scientists. The well-known hard circumstances of the self-sacrificing life of Dr Sakharov deprive him, unfortunately for all of us, from being a head of a school in this sense.

My personal vision influences also my appraisal of the achievements of Dr Sakharov. I believe that in the rank of highest human intellectual achievements and accumulation of information of vital importance, applied science is subordinate to fundamental science. In alliance with humanism and religion, fundamental science is the major contributor to the progress of humanity and also to our primary hope for surviving in the irrevocable crises of human environment and human society. The phenomenon of gravity, in its relation to space-time and causality, has been the greatest challenge to the human intelligence and ability to comprehend nature. The potentialities of an advanced theory of gravity can be enormous. So, in my personal view, no further words are required to expound the standing of Sakharov if the question "Who unravelled the mystery of gravity?" is answered: Newton, Einstein and Sakharov.

I welcome the Medvedev letter that gives an additional opportunity to draw the attention of the Soviet authorities to the urgent obligation to Sakharov across all political barriers.

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1. Medvedev, A. Zh. *Nature* 319, 93 (1986).
2. Gliner, E. B. *Nature* 318, 513 (1985).

Comet RNA shock

SIR—In view of recent correspondence on deciphering primitive evolution from bumps on ribosomes, and in the light (or blackness?) of Professor Hoyle's theory that life originates in comets, is it significant that the nucleus of Halley's comet looks exactly like the small subunit of a ribosome?

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Correction

THE British Library Lending Division is at Boston Spa, West Yorkshire. In a letter about PhD theses from Sheila M. Mould (*Nature* 319, 616; 1986), it was said to be at Leamington Spa; this was a typographical error. □

Can chance be less than zero?

An intriguing review of physical problems yielding negative probabilities may fail to demonstrate their reality, but should not be overlooked on that account.

By simple definition, as everybody knows, the values assigned to quantities representing the probability of events must be positive real numbers. How could it be otherwise? The chance that a flipped coin will come down showing heads, not tails, is roughly 0.5, the probability of the only other possible outcome is roughly the same, and the two numbers added together make exactly 1.0, which is the probability that there will be an outcome of one kind or the other. In such a context, the notion that probabilities might have negative values is entirely devoid of meaning. That is what everybody knows.

None of this has deterred Dr W. Muckenheimer of Göttingen from compiling a substantial and fascinating review of negative probability, called "extended probability" no doubt so as to avoid the charge of sensation-mongering (*Physics Reports* **166**, 337; 1986). Part of Muckenheimer's case is that if it has been useful to extend the set of natural numbers to include negative and irrational and imaginary numbers, to allow systems to have negative energy (as in some solutions of Dirac's equations) and even negative temperatures (as in laser and maser materials with artificial populations of excited states), there can be no good reason why probabilities should be sacrosanctly positive. His more tangible starting point is a puzzle which has been about in quantum mechanics since it was first identified by Eugene Wigner in 1932.

Muckenheimer comes to no particular conclusion about the meaning of negative probability. Properly, he notes that the question is almost certainly linked with other current imponderables, such as the question whether quantum mechanics requires a revision of classical causality or even of the principles of logic (as first suggested by von Neumann); tinkering with the meaning of probability may be a simpler task.

But instead of a conclusion, Muckenheimer has assembled the opinions of several of those who have contributed to the field, including refreshingly those of E.T. Jaynes of Washington University, St Louis, and M.S. Bartlett, long-since retired from the University of Manchester but plainly doing more than merely growing roses at his private address in Devon.

Wigner's conundrum arises from his attempt (with Szilard) to calculate for a general quantum system the joint probability distribution of the coordinates

which describe it and of the conjugate momenta. Although the uncertainty principle disallows the simultaneous exact measurement of these quantities, it is clear that there must be conjugately linked probability distribution functions for position and momentum.

For a system with only one degree of freedom with a position coordinate x and momentum p , for example, is a simple matter to calculate the properties of the momentum distribution function for any stationary state of the system for which the wavefunction is known, say as $u(x)$. For then the successive moments of the momentum distribution function can be calculated directly from the wavefunction as the integral over the range of x of the function $u^*(x)pu(x)$ where $*$ denotes the complex conjugate of the wavefunction and p is the momentum operator ($\hbar/i$) (d/dx).

So why not calculate the distribution function that gives the joint probability that x is within a certain interval and p the scalar value of the momentum within another? This is what Wigner did in 1932. The result was a distribution function (for one degree of freedom) $P(x,p)$ which, apart from a factor $1/(pi \cdot \hbar)$, is the integral over the dummy variable y of the quantity $u(x+y) \cdot u(x-y) \cdot \exp(2ipy/\hbar)$. The result, which is obviously generalized to systems with more than one degree of freedom, is a distribution function with some of the right properties, but which also has the disconcerting property of being negative over some parts of the range of x and p . Muckenheimer gives an interesting account of the circumstances in which the Wigner function and its many generalizations misbehave in this way.

This is not the only connection in which negative probabilities are known to occur. Relativistic generalizations of classical quantum mechanics habitually yield negative energies and, with them, functions which it is tempting to interpret as probability distributions that are capable of being negative, so much so that Dirac is quoted as believing them to be inseparable. They also crop up in field theories, as for example in schemes for accounting for the interaction between real particles by the emission and absorption of "virtual" field particles. Similar difficulties arise in the discussion of the Einstein-Rosen-Podolski paradox, the *Gedankenexperiment* designed in 1935 as a challenge to Bohr's dictum that physical variables that

do not commute with each other cannot in any circumstances be measured simultaneously, and from which the more recent discussions of Bell's inequality and Aspect's experimental tests of quantum correlations have flowed. Muckenheimer proves, by demonstration, his belief that negative probabilities signal connections with the important problems of physics.

But what do negative probabilities mean? Bartlett (who wrote a paper on negative probability in 1945) provides one simple way of fixing ideas: a convenient way of handling the probability that a biased coin will fall on one side or the other is to represent the chance that it will turn up heads as $0.50 + p$, in which case the extra variable is plainly capable of being negative as well as positive. Even so, Bartlett was able to show (in 1945) that quantities representing probabilities which can take negative values can be manipulated by most of the usual rules of the probability calculus. His view then has not changed much over the years; most of the difficulties with negative probabilities appear to arise from people's determination to follow Max Born in the interpretation of the square of the wave function as a probability density, for which he sees no physical justification.

Running through this commentary on negative probability is the opinion that, in practice, sensible people will ensure that they do not interpret probability distributions physically unless they have somehow manipulated them so as to be everywhere positive.

Other ways of squaring this circle are more ingenious. Dewdney, Holland, Kiprianidis and Vigier from the Poincaré Institute in Paris suggest that it might be worthwhile to consider negative probability distributions as the difference of two separate and presumably positive densities which may, for example, represent the distributions of particles and antiparticles respectively.

Gallantly for one who clearly wishes that somebody would give meaning to negative probability, Muckenheimer gives almost the last word to Jaynes who, while sharing the general doubt about the validity of Born's interpretation of the square of the wave equation as a probability density, makes the simple point that the onus of proof rests on those who talk about negative probability. That, unfortunately perhaps, will be the general view.

John Maddox

Vertebrate phylogeny

Are fruit bats primates?

from R. D. Martin

It is widely held that no clear characteristics define the order Primates. Instead, it has often been proposed that living primates (lemurs, lorises, tarsiers, monkeys, apes and man) form a 'phylogenetic scale' with the most primitive living mammals (insectivores) differing relatively little from the lemurs, which themselves form a graded chain with the other living primates leading to the human species at the summit. Hence, the primates are commonly defined on the basis of trends, rather than on the basis of shared diagnostic features¹. By such an approach, tree shrews are included in the Primates, as they were seen as another link in the chain, bridging the gap between insectivores and lemurs. Simpson, who formally included tree shrews in the order Primates stated that, whereas bats (order Chiroptera) can be defined on the basis of their wings, there is no simple criterion distinguishing primates². But J.D. Pettigrew has now shown that three species of fruit bats have a primate-like visual system. On this basis he suggests that fruit bats and primates shared a specific common ancestry and that bat wings evolved twice³.

The starting-point for discussions of distinctive features of the Primates has commonly been the definition proposed by Mivart⁴. This definition, however, contains several features that are probably primitive for placental mammals and few of the characters listed are reliably diagnostic of primates. This is hardly surprising, as Mivart did not believe that the living primates constitute a genuine phylogenetic assemblage. He suggested that the strepsirrhine primates (lemurs and lorises) and haplorhine primates (tarsiers, monkeys, apes and man) originated separately from ancestral placentals.

Living primates (excluding tree shrews) do, in fact, share a set of features that arguably provide a basis for clear definition^{5,6}. Provided that the tree shrews are excluded, several characteristics are found to be universal, or virtually universal, to living primates and rare or completely absent from other mammals. But many of these characteristics relate to 'soft parts', which makes it difficult to formulate a definition that also applies to fossil primates. A good example is provided by the visual system in living primates^{7,8}.

In this work, Allman^{7,8} showed that in the organization of the optic tectum in the midbrain all primates so far examined exhibit an unusual pattern that was apparently lacking from all other vertebrates. In the typical vertebrate condition, the optic tectum on one side of the brain

contains a systematic representation of the visual field viewed by the eye on the opposite side of the body (contralateral eye). In the primate condition, by contrast, the optic tectum on one side of the brain contains a systematic representation of the contralateral half of the total visual field. In other words, in the retinotectal projection of primates the visual field is bisected such that the left sector of each retina projects to the right optic tectum, and vice versa. This difference in retinotectal projection between primates and non-primates is associated with a difference in the degree of crossing-over of nerve fibres at the optic chiasma. In non-

primates, most or all of the fibres passing to the tectum are crossed (contralateral projection), whereas in primates there is an approximate balance between contralateral and ipsilateral fibres supplying the tectum on each side of the brain.

Allman linked the special retinotectal pattern to the pronounced forward orientation of the eyes found in all primates of modern aspect (that is, living and fossil primates excluding the early Tertiary Pleiadapiformes). In all of these forms, the eye-sockets (orbits) are large, forward-facing and equipped with a bony post-orbital bar produced by fusion of struts of the frontal and jugal components of the skull. Allman reasonably concluded that the ancestral primates of modern aspect possessed these features, along with the special pattern of organization of the retinotectal projection, as an adaption for improved stereoscopic vision. Although tree shrews possess a postorbital bar (a feature which has been developed convergently in several mammal groups), they lack all of the special features of the retinotectal projection found in primates.

Allman's conclusions regarding the uniqueness of the primate pattern of retinotectal projection must now be modified in the light of Pettigrew's new data on the organization of the visual system in bats³. Using electrophysiological and neuroanatomical methods, he has shown that three species of fruit bats (*Pteropus* sp.) exhibit the primate-like pattern of retinotectal organization, whereas ghost bats (*Megaderma gigas*) exhibit the typical non-primate pattern. It has long been recognized that the relatively large-eyed fruit bats or 'flying foxes' (suborder Megachiroptera) are sharply distinct from other bats (suborder Microchiroptera) and generally lack the specialized adaptations for echolocation that are typically found in the latter. As the fruit bats constitute a quite homogeneous group allocated to a single family (Pteropidae), and as *Megaderma* has one of the most developed visual systems found among microchiropteran bats, it is reasonable to conclude that the primate-like retinotectal pattern is probably characteristic of the Megachiroptera as a group but uniformly absent from the Microchiroptera. In fact, fruit bats also resemble primates in possessing relatively large, forward-facing orbits, although the postorbital bar is incomplete (see figure). It is possible, therefore, that fruit bats have developed a primate-like visual system through convergent evolution but an alternative possibility, proposed explicitly by Pettigrew, is that reorganization of the retinotectal system is a shared derived feature of fruit bats and primates, indicative of specific common ancestry. According to this interpretation, fruit bats are more closely allied to primates than (for example) tree shrews, whereas microchiropteran bats

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Skulls of a fruit bat (*Pteropus*, left) and a Madagascar lemur (*Lemur*, right). In both skulls, the orbits are rotated forwards, ensuring a large binocular field. However, the postorbital bar is incomplete in the fruit bat. The ventral views (above) show two conspicuous specializations of the fruit bat skull that are lacking in the lemur: there is a marked extension of the palate behind the last molar and a highly modified, degenerate cusp pattern on the cheek teeth. (The latter feature hinders determination of the phylogenetic relationships of fruit bats.) Note the lack of an inflated auditory bulla and the relatively small brain case in the fruit bat.

Mike Gilbert

represent a quite separate group of placental mammals.

Although it has been generally accepted that bats constitute a discrete phylogenetic unit defined by possession of wings (for example ref. 2), it is theoretically possible that the order Chiroptera is an artificial assemblage. As Pettigrew notes, either the primate-like retinotectal pattern has evolved twice among the mammals or bat wings have evolved twice. It should be noted that Linnaeus originally included bats (*Vespertilio*) in the order Primates, and Gregory suggested that primates, bats, tree shrews, elephant shrews and flying lemurs should be united in the superorder Archonta⁹. Several authors have recently revived the concept of the Archonta as a valid phylogenetic grouping. Thus, Pettigrew's suggestion merits detailed consideration.

Despite the similarities in the visual system, however, fruit bats (like tree shrews) lack several other defining features of primates of modern aspect⁶. Three examples may be cited: first, in primates, the petrosal bone plays a major part in the formation of the ventral wall of the auditory bulla, whereas both fruit bats and microchiropteran bats possess a composite bulla formed from entotympanic and ectotympanic elements (see figure). Second, primates of modern aspect are characterized by hindlimb domination, associated (except for the special case of *Homo*) with a grasping adaptation of the foot involving marked divergence of the hallux (big toe). All bats have relatively short hindlimbs and the foot typically has five parallel claw-bearing toes. Curiously, there is one bat genus with a grasping, primate-like foot, but it is a microchiropteran (*Cheiromeles*). Finally, primates exhibit a unique relationship between fetal brain and body size that is not found in bats and in the primate brain a triradiate calcarine sulcus is consistently present, whereas this sulcus is uniformly absent from bats.

Thus, any link between fruit bats and primates must be relatively tenuous. It should also be noted that the hypothesis of a phylogenetic relationship between fruit bats and primates receives no support from comparative molecular studies. Im-

munological comparisons indicate that the bats constitute a cohesive phylogenetic unit and diverged from a single ancestral stock some time after the initial radiation of the placental mammals¹⁰. Amino-acid sequence data for bat proteins are scarce at present, but available information indicates no specific relationship between any bats and primates¹¹. Hence, the present balance of evidence would seem to favour convergent evolution of the visual system in fruit bats and primates.

Discovery of a primate-like visual system in fruit bats is also significant in another respect. Cartmill, in a stimulating and thorough critique of the 'arboreal' theory of primate evolution suggested that the special developments of the pri-

mate visual system cannot be explained through arboreal life *per se* but only through visually directed arboreal predation on insects¹². The fact that forward-facing eyes and primate-like organization of the retinotectal system should have evolved in fruit-eating megachiropteran bats, rather than in insect-eating microchiropteran bats, now provides support for the modified suggestion that the primate visual system evolved in connection with feeding on both fruits and arthropods in the 'fine-branch niche' constituted by the terminal branches of trees. □

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Nitrogen fixation

Gaseous diffusion in cyanobacterial heterocysts

from Frank Minchin

THE nitrogenase enzyme of aerobic nitrogen-fixing microorganisms is very sensitive to oxygen damage, so how is the enzyme protected while an adequate inflow of gaseous nitrogen and oxygen is maintained? One solution is to carry out nitrogen fixation within a physical barrier to slow down the rate of gas diffusion such that virtually all the oxygen crossing this barrier is consumed in respiration. Cyanobacteria produce specialized cells called heterocysts with thickened cell envelopes which are considered to be an adaptation to this strategy (Fay, P., Stewart, W.D.P., Walsby, A.E. & Fogg, G.E. *Nature* **220**, 810; 1968). Two questions concerning heterocysts, however, have long remained unanswered: is the permeability of the envelope low enough to allow it to function as an oxygen barrier; and, if so, does the envelope also restrict the influx of nitrogen so that it limits fixation? Answers to these questions have now been provided by A.E. Walsby (*Proc. R. Soc. B* **226**, 345; 1985).

To study the permeability of heterocyst envelopes Walsby has developed an elegant technique that exploits the presence of gas vacuoles throughout cyanobacteria, for example *Anabaena flos-aquae*. These vacuoles consist of stacks of hollow cylindrical vesicles that are extremely permeable to gas (Walsby, A.E. *Bact. Rev.* **36**, 1; 1972). When the hydrostatic pressure around the cyanobacterial fila-

ments is increased these vesicles tend to collapse, but this can be prevented by an increase in internal pressure resulting from diffusion of gas into the vesicles. Thus, collapse of the vesicles (see figure) will occur if the rate of increase in external pressure is faster than the rate of gas diffusion. This latter rate is a function of the permeability of the vesicle-containing cell. Walsby calculated this permeability by determining the critical rate of pressure increase required to collapse 50 per cent of the vesicles.

This critical rate for heterocysts is about 100 times slower than for vegetative cells and the envelopes have a mean permeability coefficient of 0.3 s^{-1} . From this Walsby calculated that the maximum influx rate per heterocyst would be $26.7 \times 10^{-18} \text{ mol s}^{-1}$ for nitrogen and $9.6 \times 10^{-18} \text{ mol s}^{-1}$ for oxygen. The difference in rates for the two gases is mainly a reflection of their different solubilities in water. The mean nitrogen-fixation rate per heterocyst was calculated as $5.9 \times 10^{-18} \text{ mol s}^{-1}$, and derived rates of oxygen consumption ranged from 7.7 to $15 \times 10^{-18} \text{ mol s}^{-1}$. Thus, the permeability of the heterocyst envelope is

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Light micrographs (courtesy of A.E. Walsby) of a filament of *Anabaena flos-aquae* ($\times 1,260$) (a) before and (b) after exposure to a pressure of 0.7 MPa. Note the disappearance of gas vacuoles from all cells in (b). The heterocyst (centre) is distinguished by its thickened envelope and, in b, by the dark plugs at the ends of the connecting pores.

1. Le Gros Clark, W.E. *The Antecedents of Man* (Edinburgh University Press, 1971).
2. Simpson, G.G. *Bull. Am. Mus. Nat. Hist.* **105**, 415 (1955).
3. Pettigrew, J.D. *Science* **231**, 1304 (1986).
4. Mivart, St G. *Proc. Zool. Soc. Lond.* **1873**, 484 (1873).
5. Martin, R.D. *Man* **3**, 377 (1968).
6. Martin, R.D. in *Major Topics in Primate and Human Evolution* (eds Wood, B.A., Martin, L.B. & Andrews, P.J.) 31 (Cambridge University Press, 1986).
7. Allman, J.M. *Progr. Physiol. Psychol.* **7**, 1 (1977).
8. Allman, J.M. in *Primate Brain Evolution* (eds Armstrong, E. & Falk, D.) 13 (Plenum, New York, 1982).
9. Gregory, W.K. *Bull. Am. Mus. Nat. Hist.* **27**, 3 (1910).
10. Cronin, J.E. & Sarich, V.M. in *Comparative Biology and Evolutionary Relationships of Tree Shrews* (ed. Luckett, W.P.) 293 (Plenum, New York, 1980).
11. Goodman, M. et al. in *Macromolecular Sequences and Evolutionary Biology* (ed. Goodman, M.) 115 (Plenum, New York, 1982).
12. Cartmill, M. *Science* **184**, 436 (1974).

more than adequate to allow the required nitrogen influx, but oxygen influx is restricted such that respiration can maintain the internal concentration close to zero.

This 'fine tuning' of the heterocyst envelope permeability could, however, lead to problems with oxygen. If oxygen enters the heterocysts at a constant rate then this must be balanced by a constant rate of respiration. A reduced supply of respiratory substrate from the adjacent vegetative cells (for example, in the dark) would result in a build up of internal oxygen sufficient to cause nitrogenase damage. Furthermore, in daylight sufficient ATP for nitrogen fixation can be produced by cyclic photophosphorylation (Stewart, W.D.P. *A. Rev. Microbiol.* **34**, 497; 1980), but in the dark this ATP is generated by oxidative phosphorylation. This latter process requires a rate of oxygen influx 3–4 times greater than the flux of nitrogen needed for fixation, but Walsby's measurements suggest an oxygen influx less than half that of nitrogen. Thus, the rate of oxygen supply at night could either limit nitrogen fixation, if carbohydrate supply was adequate, or threaten nitrogenase, if the carbohydrate supply was reduced.

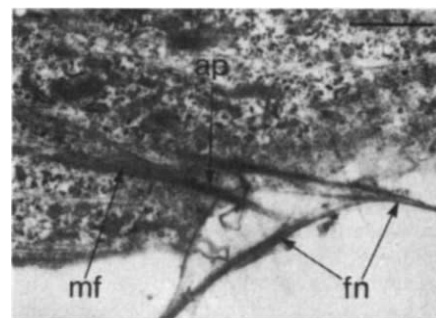
A solution to this problem would be a controlled, variable inflow of gases into the heterocysts. It is doubtful if this could be achieved by altering the permeability of the rigid, three-layered envelopes. A small inflow of gas into the heterocysts could occur through the pores that connect them to vegetative cells but these pores are occluded by terminal plugs (see

figure) that may produce a fixed resistance to gas diffusion. Any variation in the distance between the heterocyst and the air–water interface, however, will alter the diffusion resistance created by the water, and thus a diurnal variation in depth for the aquatic cyanobacteria would produce an additional control of the influx of gases. The significance of such a regulatory mechanism requires further investigation, as does the situation for cyanobacteria growing on moist soil.

The confirmation of the heterocyst envelope as an effective barrier to oxygen diffusion should stimulate study of other aerobic nitrogen-fixing systems that have the same general strategy for oxygen regulation, such as the vesicles of *Frankia* and the nodules of leguminous plants. But it is unlikely that these other structures will prove as amenable as cyanobacterial heterocysts. Indeed, the site and nature of the diffusion barrier in legume nodules is still a matter of conjecture.

Walsby's work is also of interest to those seeking to extend the range of nitrogen fixation within agriculturally important crops. It is generally recognized that the creation of novel root-based systems would involve a substantial redistribution of carbohydrate within the plant. An alternative strategy is a leaf-based nitrogenase system closely coupled to photophosphorylation; heterocysts appear to be the ideal blueprint for thinking along these lines. □

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Electron micrograph showing colinearity of submembrane actin microfilaments (mf), adhesion plaques (ap) and extracellular fibronectin-like fibrils (fn) in a chick embryo fibroblast. Scale bar, 1 μ m.

studying the interactions of molecules that form rapidly dissociating complexes.

Earlier, this technique was only used to study the interactions of small ligands with receptors, but Horwitz *et al.* have adapted the method for large macromolecular interactions. In the traditional method, a gel filtration column is pre-equilibrated with a potential ligand before mixtures of the receptor and ligand are run through it, which ensures continuous receptor occupancy in receptor–ligand complexes. Horwitz *et al.* find that the elution profile of CSAT is significantly shifted when the complex is run both with talin and with a mixture of talin and fibronectin, showing that these molecules had bound to each other. Importantly, the binding of fibronectin to the CSAT–talin complex is inhibited by a tetrapeptide sequence derived from the fibronectin molecule that is specifically involved in cell–matrix adhesion. The tetrapeptide does not affect the formation of the talin–CSAT complex, showing that CSAT has two separate binding sites for fibronectin and talin. Another protein involved in actin–membrane interactions is vinculin, which, like talin, is found at sites of cell adhesion. Vinculin and talin will form a complex *in vitro*⁵, so Horwitz *et al.* investigated another link in this transmembrane chain by incubating CSAT with both talin and vinculin, and identifying a further complex of these three proteins by gel filtration. The authors were able to show that CSAT does not bind directly to vinculin; rather, CSAT and vinculin bind separately to a large (M_r 190,000) domain of the talin molecule.

The CSAT receptor is found on most chick embryo cells. Monoclonal antibodies to CSAT reveal differences in the involvement of the receptor in cell–substratum adhesion. Curiously, cardiac fibroblasts are rather insensitive to CSAT antibodies, but tendon fibroblasts and skeletal myoblasts are rapidly detached from fibronectin-coated surfaces by low concentrations of antibody⁶. These results suggest that a multiplicity of adhesive mechanisms are operational, perhaps involving other receptors.

Cell biology

Finding the missing links

from Julian Heath

EARLY embryonic development is characterized by wide-ranging cell migrations. The tractive forces for movement are provided by interactions of the proteins actin and myosin in the cellular cytoskeleton and are applied through adhesions to a substratum composed of an extracellular matrix of collagen, fibronectin, laminin and other proteo- and glycosamino-glycans. Fibroblast cells in culture are known to secrete matrix molecules that bind to the cell surface in fibrillar arrays (see figure) but the adhesion of cells to the matrix becomes defective on transformation. How are these two compartments, the cytoskeleton and the extracellular matrix, linked at their interface, the plasma membrane? Research in this area has been somewhat polarized; some groups study matrix molecules and their receptors and others attempt to unravel the interactions in the cytoskeleton. Now, in a paper published

on page 531 of this issue¹, Horwitz and co-workers have brought the two fields closer together with the discovery that a membrane protein complex, CSAT, is a receptor for both fibronectin and for talin, a cytoskeletal protein specifically located at sites of the cell–substratum adhesion where actin filaments meet the plasma membrane².

CSAT, a complex of three integral membrane proteins and with a relative molecular mass (M_r) of 140,000, can be isolated from chick fibroblasts by a monoclonal antibody³. Earlier studies showed that CSAT is a receptor for fibronectin and laminin⁴ with a rather moderate affinity of 10^{-6} M. Attempts to identify other ligands for CSAT have proved difficult, apparently because of weak or rapidly exchanging equilibria. To avoid this problem, Horwitz and co-workers exploited the technique of equilibrium gel filtration, which is particularly suitable for

Location of fibronectin, membrane proteins and cytoskeletal proteins in regions of actin-membrane interaction

	Matrix		Membrane proteins			Cytoskeleton		
	FN	CSAT	JG22	30B6	ID7	TAL	VIN	AA
Cultured fibroblasts								
Ruffles	—	+				+		
Adhesion plaques (centre)	—	—	—	+		+	+	+
Close contacts and periphery of plaques	+	+	+					+
Extracellular matrix contacts	+		+					+
Intercellular contacts (all)			+/-	+			+	
Cleavage furrow				+				+
Cultured neurones ¹⁴								
Cell body, axons and growth cones		+						
Gizzard smooth muscle								
Membrane-dense plaques	—		—	+		+	+	+
Other sites	+		+					
Cardiac muscle								
Intercalated discs					+		+	+
Eye lens fibres								
Adherens junctions					+		+	
Intestinal epithelial cells								
Zonulae adherens			+				+	+

Data collated from cited references. FN, fibronectin; TAL, talin; VIN, vinculin; AA, α -actinin. Membrane receptors are identified by the monoclonal antibody code.

Recently, other adhesion proteins, all with similar relative molecular mass (about 140,000) have also been identified using monoclonal antibodies; indeed, the evidence from gel electrophoresis and from immunocytochemistry does suggest that they are closely related. In the table, the cellular distribution of CSAT and three other proteins are listed and compared with the distribution of three cytoskeletal proteins known to be involved in actin-membrane binding: talin, vinculin and α -actinin.

In cultured fibroblasts⁷, the CSAT receptor is more dense on the membrane of actin-containing marginal ruffles where talin is also specifically enriched. But the co-localization of CSAT and cytoskeletal proteins is less clear in the adhesion plaques: here, talin and vinculin are components of the cytoplasmic side of the membrane in the central regions of the plaques, whereas CSAT is found surrounding the plaque in a needle's-eye configuration. However, the morphology confirms the biochemistry at sites called extracellular matrix contacts where sub-membraneous bundles of actin co-align with extracellular fibrils of fibronectin (see figure); CSAT is located here. These types of contact are probably not impor-

tant for cell motility *in vitro* but they could be analogous to cell-matrix contacts in the embryo.

Of the other membrane proteins listed in the table, one, a fibronectin receptor complex isolated by the monoclonal antibody JG22 (ref.8), has similar if not identical properties and distribution to CSAT. And another antibody, 30B6, also recognizes a complex similar to CSAT but differs in that its antigen is found in the central regions of fibroblast adhesion plaques⁹. Lastly, a M_r 135,000 membrane protein¹⁰ which co-localizes with vinculin but not with talin differs from the others in that it is found at cell-cell but not cell-

substratum junctions.

Horwitz and colleagues provide cogent biochemical evidence for the existence of a transmembrane chain of molecules leading from the extracellular matrix, through the membrane receptor CSAT, to talin, vinculin and, directly or indirectly, to actin filaments in the cytoskeleton. How are these chains forged? Are they made *de novo* or are parts pre-assembled before the cell makes an adhesive contact? Direct observations of adhesion formation in moving cells indicate that possible precursor structures containing actin¹² and vinculin¹³ may be formed before an adhesive plaque is made.

Furthermore, the presence of vinculin and talin at sites of cell-substratum adhesion is not dependent on the presence of actin filaments¹¹. The signal that causes vinculin and talin to bind to CSAT may be crosslinking of the receptors with their ligand, fibronectin; in the undoubtedly similar phenomenon of patching, the aggregation of membrane proteins by antibodies of widely differing specificities also leads to a transmembrane association of the antibody-protein complexes with the cytoskeleton.

We still do not know what other membrane proteins and cytoplasmic actin-binding proteins such as α -actinin are necessary to link complexes like CSAT to actin microfilaments at sites of adhesion. Clearly it is important to discover how receptor complexes are linked to actin microfilaments, in what order these molecular interactions occur and why such linkages fail to develop or operate properly in tumour cells. □

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Evolution of antibodies

The road not taken

from Walter Gilbert

THE immune system of mammals has evolved to cope with a virtually limitless antigenic world. Each new antigen entering the body elicits thousands of different antibodies capable of binding it and ensuring its elimination. Subsequent challenge with the same antigen produces antibodies that are different again, having usually a higher affinity for the antigen and a different mode of eliminating it. But the diversity of the immune response as well as its maturation depends on a mechanism for recombining different segments of DNA in the somatic cells that produce the antibodies. How did this ability of the immune system to match the diversity of the outside world arise? K.R. Hinds and G.W. Litman, whose paper appears elsewhere in this issue (*Nature* 320, 546; 1986) have

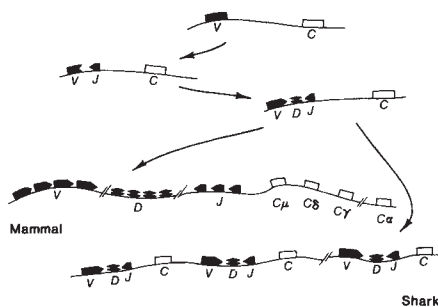
attempted to trace the evolution of immunoglobulin diversity by looking at the immunoglobulin genes of the horned shark, whose immunoglobulin diversity is much more limited than that of mammals, although the structure of the protein is essentially the same.

In mammals, the immunoglobulin structure that binds an antigen is created by juxtaposing two variable regions, one from a two-domain light chain and one from a four- or five-domain heavy chain (reviewed by Tonegawa, S. *Nature* 302, 575; 1983 and Hood, L. *et al. Cell* 40, 225; 1985). Each of these domains represents a common shape, the 'immunoglobulin fold', and is encoded by a single exon (see figure). The amino-terminal domain of each chain, the variable domain, is

1. Horwitz, A.F. *et al. Nature* 320, 531 (1986).
2. Burridge, K. & Connell, L. *Cell Motil.* 3, 405 (1983).
3. Neff, N.T. *et al. J. Cell Biol.* 95, 654 (1982).
4. Horwitz, A.F. *et al. J. Cell Biol.* 101, 2134 (1985).
5. Burridge, K. & Mangeat P. *Nature* 308, 744 (1984).
6. Decker, C. *et al. J. Cell Biol.* 99, 1398 (1984).
7. Damsky C. *et al. J. Cell Biol.* 100, 1528 (1985).
8. Chen W.-T. *et al. J. Cell Biol.* 100, 1103 (1985).
9. Rogalski, A.A. & Singer, S.J. *J. Cell Biol.* 101, 785 (1985).
10. Geiger, B. *et al. J. Cell Biol.* 101, 1523 (1985).
11. Avnur, Z. *et al. J. Cell Biol.* 96, 1622 (1983).
12. DePasquale, J.A. & Izzard, C.S. *J. Cell Biol.* 101, 411a (1985).
13. Geiger, B. *et al. J. Cell Biol.* 99, 83s (1984).
14. Bozyczko, D. & Horwitz, A.F. *J. Neurosci.* (in the press).

specialized for antigen binding. The variable domain of the heavy chain is created by bringing together three different regions along the DNA molecule to create a complete exon. These elements are a variable (*V*) segment, a diversity (*D*) segment and a joining (*J*) segment. One element of each class is combined by the deletion of the intervening DNA regions to create a variable-domain exon. The first source of variation is a combinatorial choice between several hundred different *V*s, some twenty different *D*s and four different *J*s. The second source of variation arises from the junctional diversity created as new codons are formed by imprecise recombination as these elements are fused together and a further diversification around the *D* segment where the enzyme terminal transferase inserts new sequences (Alt, F.W. & Baltimore, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4118; 1982). After transcription the final variable-domain exon is then spliced to the exons for the domains of the constant region (the *C* region) for the immunoglobulin M (IgM) heavy chain. The heavy chain then combines with a light chain, whose variable domain is made up of a *V* and a *J* portion, again the product of a combinatorial and a junctional diversity, to produce a functional immunoglobulin molecule.

There are two further stages of specialization in the mammalian immune system after the first challenge with antigen has brought forth the primary (IgM) antibody response. The first is immunological memory, which is mediated by cells that have created new molecules with the original variable-region exon now attached to different sets of constant-domain exons. This produces the antibodies of the secondary response — IgG, IgA or IgE. During



Schematic representation of the evolution of the immunoglobulin genes. The original exon for the *V* domain is broken up twice during its evolution by the introduction of excision sequences. The boxes marked *C* represent sets of exons for the constant regions; *C μ* , *C δ* , *C γ* and *C α* represent the sets of exons for the heavy chains of IgM, IgD, IgG and IgA, respectively. The final gene clusters in mammals and in sharks spread across several million base pairs. For a gene to be expressed the intervening DNA between a *V* and a *D* and that *D* and a *J* excises in a cell lineage, introducing a junctional diversity, and then that final gene is transcribed into RNA and the mature *V* exon is spliced to the *C*-region exons.

the secondary response, through stimulation and selection of the cells that can generate these antibodies, somatic mutation modifies the variable region, refining the binding of the antibody to the antigen. Of these three mechanisms for the generation of diversity: combinatorial joining of subdomains; junctional diversification; and somatic mutation, which came first in evolutionary history?

Hinds and Litman have sought the answer to this question by investigating the heavy-chain genes of the horned shark, an elasmobranch. Sharks diverged from the vertebrate line before the bony fish, separating some 450 million years ago from the line that leads to the mammals. They do not mount a secondary immune response: rechallenge with the antigen produces only the primary response once again. And their immunoglobulin heavy-chain genes have an organization entirely different from that of mammals. Their genes are structured as a single *V* region linked to a single *D* region linked in turn to a single *J* region, all of these linked to a single set of *C*-region exons (see figure). This entire pattern is then reiterated many times. For one of these antibody genes to function, the intervening DNA is deleted between a neighbouring *V* and *D*, and that *D* and its *J*, to create the *V-D-J* exon, which then is connected by transcription and splicing to its corresponding constant domains. Diversity in the horned shark has two sources: inherited diversity from the many copies of the (*V+D+J*)+*C* sets, each one of which has drifted individually; and the junctional diversity on their assemblage. But the shark lacks the combinatorial amplification that is created by the match-

ing of different *V*s, *D*s and *J*s.

This alternative gene structure shows that at the moment of divergence of the two lines, 450 million years ago, the parent would have had a single copy of a (*V+D+J*)+*C* gene. The only variability in the original antibody molecule would have been created by junctional variation as the *V*, *D* and *J* regions fused together. Down one evolutionary road this entire unit was duplicated and reduplicated many times, developing new antibody specificities by drift after that. Down the road that leads to mammals, the parts of this unit — the *V* segments, the *D* segments and the *J* segments — were separately duplicated, creating still greater possibilities through the combination of different regions. Duplication of the constant region gave rise to the different constant genes now used in the primary and secondary responses, and thus provided an opportunity for the evolution of long-lived immunological memory and the somatic polishing of the immune response.

Although the ancestral chordates had simple antibody genes, they still had a complicated immune system in that they had both variable heavy and light chains for antibodies and presumably also the variable chains that comprise the antigen receptors of the T cells that are responsible for cell-mediated immunity and the control of the immunoglobulin response. The immune system of those organisms had evolved a very long distance from the first introduction of a generator of diversity. One imagines the immune system began first as the reiteration of a basic unit, the immunoglobulin fold, repeated on multiple exons and used just for its general binding property. Diversification began with the insertion of a DNA element into a critical part of one immunoglobulin exon in such a way as to divide it into a *V* and a *J* segment. The role of this sequence was to be triggered to excise itself inaccurately from the DNA, and thus to provide junctional variation when it left the DNA, creating in each new cell a new complete variable-region exon with an altered amino-acid sequence arising at the point of excision (Sakano, H. *et al. Nature* **280**, 288; 1979). This created the first variation, similar to the variation we currently see in the light chains. After a further duplication, the introduction of another excision sequence created the primordial heavy chain capable of joining a *V* to a *D* and a *D* to a *J*. These primordial genes had to duplicate again to generate T-cell and B-cell receptors. Throughout much of evolutionary history, junctional diversity held sway as the sole creator of immunoglobulin variation. □

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100 years ago

The Sunrise Shadow of Adam's Peak, Ceylon

SOME of the phenomena of the shadow of Adam's Peak in the early morning have been remarked by almost every traveller who has visited this island. The mountain rises to a height of 7,352 feet as an isolated cone projecting more than 1,000 feet above the main ridge to which it belongs. The appearance which has excited so much comment is that just after sunrise the shadow of the Peak seems to rise up in front of the spectator, and then suddenly either to disappear or fall down to the earth. I determined to attempt the discovery of the true nature of this appearance. Suddenly, at 6.30 a.m., the sun peeped through a chink in the eastern sky, and we saw a shadow of the Peak projected on the land. Twice this shadow appeared and vanished as cloud obscured the sun, but the third time we saw what has apparently struck to many observers. As a mass of vapour drove across the shadow, the condensed particles caught the shadow, and were also large enough to form a bow. As the vapour blew past, the shadow fell to its natural level — the surface of the earth.

From *Nature* **33** 532, 8 April 1986.

DNA structure

Bent molecules — how and why?

from David Lilley

In the not so distant past, DNA molecules were thought of as rather rigid, straight entities which only bent with reluctance. However, it must be possible to distort the path of DNA quite severely, for it is possible to pack long DNA molecules into rather cramped quarters, such as the volume of a phage head. Recent work with the DNA of kinetoplast circles isolated from trypanosome mitochondria has revealed that DNA can undergo bending which is intrinsic to its sequence. Hydrodynamic and electrophoretic experiments^{1,2}, previously discussed in these columns³, demonstrated that linear fragments of kinetoplast DNA possessed quite abnormal geometry, which was attributed to bending, or possibly to flexibility. On page 501 of this issue⁴ Koo, Wu and Crothers, in a veritable *tour de force*, provide a detailed mechanism for the bending. The implications of this and other recent studies for DNA structure are fascinating.

Using a novel circular permutation gel electrophoresis assay, which like all the best techniques in science is all the more powerful for its simplicity, Wu and Crothers⁵ localized the bending locus to a

range; and Hagerman² has used a significantly modified application of the Dickerson–Calladine⁹ rules to calculate the trajectory of the kinetoplast bending sequence. (These rules were derived by inspection of a crystal structure of B-DNA¹⁰, and the essential feature is a deformation of the helix in response to a stereochemical 'clash' of consecutive purine bases on opposite strands, thereby opening one groove and compressing the other.)

A quite different model was proposed by Levene and Crothers¹¹, in which the requirement that the DNA remains in the B-conformation is relaxed. PolydA.polydT, the non-alternating polymer with A on one strand T on the other, is known to be structurally abnormal (for example, it resists reconstitution into nucleosomes) and Arnott and co-workers have proposed a non-B conformation where the two strands have significantly different conformations¹². They termed this structure H (heteronomous)-DNA. The basis of the Levene–Crothers model is that the A_n runs adopt a non-B conformation, and that there is a marked change in direction of the helix axis at the junction between B and non-B sections. One piece

GAAT TCCCAAAAATGTCAAAAATAGGCCAAAAATGCCAAAAATCCCAAAC
 ↑
 bend centre
 The base sequence at the bending locus of kinetoplast DNA

region of the DNA distinguished by recurring runs of adenosines (see figure). Subsequently, other fragments of DNA which migrated anomalously slowly in gels, and which had similar runs of adenosine (A_n, where *n* is the number of residues) were isolated, such as the replication origin of phage λ (ref. 6), and the *hisR* gene of *Salmonella typhimurium*⁷. In each case there is a correlation between electrophoretic retardation and repeated A_n runs, leading to the suggestion that A_n sequences deform the path of the DNA helix by some mechanism.

The mechanism of bending has been the subject of some debate. Broadly speaking there are two classes of proposals, which depend on whether or not the DNA remains in its normal B-conformation (although this is becoming continually harder to define with precision). Two models have been suggested where the DNA is essentially B-like throughout. Trifonov and Sussman⁸ proposed that the dinucleotide ApA would open to form 'wedges' by a combination of tilt and roll, and various estimates of this deviation from parallel geometry have been attempted, giving values in the 5–15-degree

of evidence in favour of this model is that the electrophoretic anomaly of these bent fragments disappears as the temperature is raised higher than about 45°C, where presumably the novel A_n conformation reverts to B structure¹³.

Whichever type of model is correct, one requirement for the establishment of a planar bend, as opposed to a random trajectory or a superhelix, is that each component bend should be added productively. This requires that the sequence elements responsible for each 'mini-bend' must repeat with the helical periodicity of the DNA, that is, 10 to 11 base pairs (bp). Diekmann and Wang¹³ subjected the sequence of a 410-bp kinetoplast DNA fragment to autocorrelation analysis, and observed, as had others by inspection, a strong harmonic component with a period of around 11 bp. This was true even for A_n where *n* takes small values, from 2 to 4, but they noted that longer A_n runs were most associated with the regions possessing greatest bending. This suggests that perhaps longer stretches of oligo (A) are required in order to stabilize the alternative conformation required in the junction model.

To examine the sequence requirements for bending systematically, a new approach has been taken that is dependent on the synthesis and ligation of oligonucleotides of defined sequence^{4, 14, 15}. In this manner sequences may be constructed and examined at will, rather than having to rely on analysis of natural sequences. The essential features of such an experiment are that an oligonucleotide comprising about one helical turn, and which contains a putative bending sequence, is self-ligated to generate a ladder of up to twenty or more oligomers. These are electrophoresed in a gel and their mobility compared with normal DNA fragments. If the sequence induces bending, then the more monomers that become ligated, the more pronounced is the bending, and so we see an increased electrophoretic retardation with oligomer length. In a variation of this experiment, Trifonov and colleagues¹⁶ recently demonstrated that a similar sequence containing two helical periods could be ligated into a circle of around 140 bp, a feat that would be very difficult for normal DNA.

The synthesis, ligation and gel electrophoresis approach has been used to highlight further the crucial importance of phasing the A_n sequence with a 10- or 11-bp period. The paper in this issue⁴ uses these methods to explore the mechanism of bending in detail. The behaviour of a series of (A_nN_k)_n oligonucleotides (where N is an unspecified nucleoside) was examined in the bending assay, as a function of *k*. A₅N₅ exhibited the greatest retardation, with A₅N₆ as a respectable second and all others non-starters. These data, especially when reinforced by some subtle temperature effects, confirm the requirement for phasing.

The length of the A block was investigated. Six bases were optimal and three insufficient, supporting the autocorrelation analysis of the natural sequences. Furthermore, interruptions of the A block, even by the fellow purine guanine, could not be tolerated. The collective

1. Marini, J.C., Levene, S.D., Crothers, D.M. & Englund, P.T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7664 (1982).
2. Hagerman, P.J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4632 (1984).
3. Widom, J. *Nature News and Views* **309**, 312 (1984).
4. Koo, H.-S., Wu, H.-M. & Crothers, D.M. *Nature* **320**, 501 (1986).
5. Wu, H.-M. & Crothers, D.M. *Nature* **308**, 509 (1984).
6. Zahn, K. & Blattner, F.R. *Nature* **317**, 451 (1985).
7. Bossi, L. & Smith, D.M. *Cell* **39**, 643 (1984).
8. Trifonov, E.N. & Sussman, J.L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3816 (1980).
9. Calladine, C.R. *J. molec. Biol.* **161**, 343 (1982).
10. Wing, R.M., et al. *Nature* **387**, 755 (1980).
11. Levene, S.D. & Crothers, D.M. *J. biomolec. Struct. Dyn.* **3**, 429 (1983).
12. Arnott, S., Chandrasekaran, R., Hall, I.H. & Puigjaner, L.C. *Nucleic Acids Res.* **11**, 4141 (1983).
13. Diekmann, S. & Wang, J.C. *J. molec. Biol.* **186**, 1 (1985).
14. Hagerman, P.J. *Biochemistry* **24**, 7033 (1985).
15. Hagerman, P.J. *Nature* (in the press).
16. Ulanovsky, L., Bodner, M., Trifonov, E.N. & Choder, M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 862 (1986).
17. Frederick, C.A. et al. *Nature* **309**, 327 (1984).
18. Drew, H.R. & Travers, A.A. *J. molec. Biol.* **186**, 773 (1985).

effect of these results is to demonstrate the importance of a phased block of A_n , where n must be a significant length, and argue for the junction model. They exclude simple flexibility, although anisotropic flexibility which leads to a time-averaged net bending may still be a possibility. The dinucleotide wedge model appears to be excluded in this system by a careful analysis of the number of such steps, and whether they should add in a constructive way. Koo *et al.*⁴ also ask whether one junction is more important than the other. If the vital element in creating the bend is a non-B 5'- A_n -3' section, then there need not be equal bending at each end. By making sequences in which the 5' ends are phased with the helix while the 3' ends alternate between 7- and 13-bp periodicity, and others where the 3' junctions are phased correctly, the authors could show that the 3' junction is the more important contributor to the overall bending.

The next stage in gaining a full understanding of the bending mechanism must be to establish both the conformation of the A_n runs and that of the junction with B-DNA. In the meantime, what is the role of DNA bending? We should probably make a distinction between bent and bendable DNA. Kinoplast DNA has a sequence which gives rise to intrinsic bending. Other sequences may be normally straight, but are relatively easily bent in response to the binding of a protein. Wu and Crothers⁵ demonstrated that the DNA upstream of the *lac* operon becomes bent on binding the cyclic AMP receptor protein (CAP). It will come as no surprise if it is found that other *trans* acting factors, either prokaryotic or eukaryotic, bend their cognate DNA. The restriction enzyme *EcoRI* appears to kink and bend its substrate on binding to the GAATTC

recognition sequence¹⁷.

Sequence-directed bending of the kinoplast DNA may be an extreme example of a more general bending that most DNA molecules are required to undergo. We have already mentioned DNA packaging in viruses. Compaction of eukaryotic DNA around the nucleosome core is another example. How may this be achieved for essentially random sequence DNA?

The answer seems to be that although the sequence itself may be random, the structure adopted is not, and forms to optimize the bending potential of the DNA. Drew and Travers¹⁸ took a 169 bp piece of bacterial DNA and circularized it. By careful analysis of cleavage frequencies by the nuclease DNase I, they were able to deduce which sequences lay on the 'inside' of the circle, and which lay 'outside'. Two points emerged: first, that there was indeed a defined inside and outside, rather than a population of all possible orientations; and second, that runs of A and T are positioned so that their minor grooves face in, while the less compressible G- and C-region minor grooves face outwards. Thus, the structure that is formed is the one that best optimizes these preferences.

The authors went on to show that this DNA reconstitutes a nucleosome retaining this same inside and outside, which suggests that exact nucleosome positioning of DNA is governed by the requirement for placing the most easily bent sequences at the point in the nucleosome with the smallest radius of curvature. It is clear that the significance of all these studies extends far beyond the function of an obscure suborganelle of a parasite. □

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Ethnic differences

Variation in human testis size

from Jared M. Diamond

THE potential harvest from studies of human testis size, a subject that has received little systematic investigation, is indicated in a paper by R. V. Short¹, who documents variations between ethnic groups which could be correlated with the incidence of dizygotic twins and breast cancer.

Although measurements of testis size by orchidometry in living subjects are difficult to standardize, they suggest smaller testes in Japanese and Korean men than in Caucasians. Weighing at autopsy is more accurate and showed that the size was two-fold lower in two Chinese samples compared with a Danish sample (see figure). Differences in body size make only a slight contribution to these values. Could the testis size variation be a Y-chromosome

trait lacking female correlates, as already documented for mice²? Interspecies differences in testis size among man and apes do fit this pattern, differences in the relative size of ovaries being negligible. In these species large testis size correlates with, and was probably selected by, two factors: high copulatory frequency; and high probability that a female will mate with several males during one ovulatory cycle³. However, evidence that these factors vary between human populations is lacking. An explicit test revealed no relation between testis size and copulatory frequency in Korean men⁴.

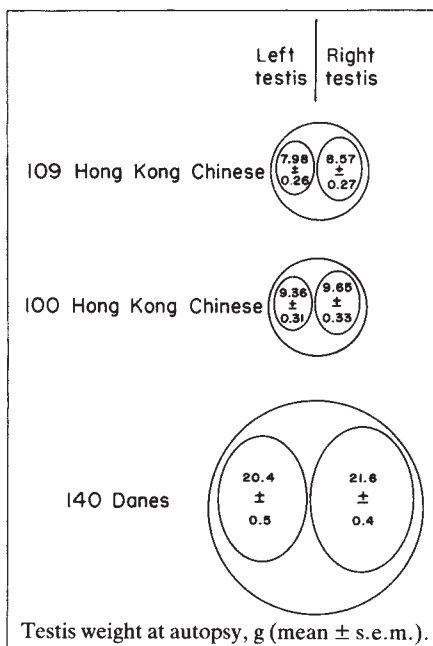
Alternatively, could the variation in human testis size be an autosomal trait with correlates in females? If male mice are

Dizygotic twinning rate per 1,000 births (from refs 1 and 7)

Population	Ethnic group	Locality	Dizygotic twinning rate
Asians	Japanese	Hawaii	2.2
	Japanese	Japan	2.3
	Chinese	Formosa	1.4
	Chinese	Hawaii	2.1
	Chinese	Malaya	2.8
	Chinese	Singapore	4.1
	Chinese	Hong Kong	6.8
	Malays	Hawaii	2.2
	Malays	Manila	2.7
	Malays	Malaya	5.2
	Hawaiians	Hawaii	3.9
	Koreans	Korea	5.1
	Koreans	Korea	5.8
	Koreans	Korea	7.9
Indians		Bombay	6.8
		Bangalore	7.3
		Calcutta	8.1
Caucasians	Europeans	Spain	5.9
	Europeans	France	7.1
	Europeans	Switzerland	8.1
	Europeans	Holland	8.1
	Europeans	West Germany	8.2
	Europeans	Norway	8.3
	Europeans	Sweden	8.6
	Europeans	Britain	8.9
African blacks	Bantu	Johannesburg	16.0
	Bantu	Leopoldville	19.0
	Yoruba	Ibadan	40.0
	Yoruba	Ilesha	49.0

selected for large or small testes, the females of these strains have correspondingly high or low ovulation rates and vice versa; similar results apply to sheep^{5,6}. As a correlate of ovulatory rate in human females, Short examined the frequency of dizygotic twin births (see table). Such births are under genetic control, apparently as an autosomal recessive⁷. The dizygotic twin frequency proves indeed to be lower in Asians (average value for 14 populations, 3.9 per 1,000 births, including 4 populations transplanted to Hawaii), than in Caucasians (average for 8 populations, 7.9 per 1,000 births). All but 2 of the 14 Asian values were less than any of the Caucasian values. The differences in dizygotic twin frequency, and presumably ovulation rate, are in the same direction as the differences in testis size. The frequencies of dizygotic twins are even higher (up to 49 per 1,000 births) among African blacks (see table).

The selective factor responsible for this parallel variation in men and women is open to speculation. One suggestion is that there should be strong selection against dizygotic twins in populations of small stature and slender build, because of increased maternal and infant mortality, regardless of whether those body traits are genetically or nutritionally determined. By this reasoning, small testis size in Asian men would be a by-product of selection against dizygotic twins in Asian women, assuming that testis size and ovulation rate are genetically linked in humans as they



are for mice and sheep.

Might these dizygotic twin frequencies have any detectable correlates in the whole female population? At first, one might expect not, because even the highest recorded frequency (Yoruba women) is only 4.9 per cent. However, the probability that a single ovulation with intercourse will result in a live birth is only $\frac{1}{4}$ for humans⁸. Thus, only one-sixteenth of double ovulations should result in births of dizygotic twins, and the frequency of double ovulations should vary among human populations up to $16 \times 4.9 = 78$ per cent. Therefore, correlates of dizygotic twinning in the population as a whole may exist.

One possible candidate for the correlate is hormone levels, as development of two follicles doubles oestrogen production during the follicular phase of the menstrual cycle. There are indications that levels of oestradiol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are higher in mothers of dizygotic twins than of singletons^{9,10}. Yoruba women, with the world's highest frequency of dizygotic twins, have higher FSH and LH levels at the time of ovula-

tion than do Japanese women, who have the lowest frequency of dizygotic twins¹¹.

This variation in female hormone levels may contribute to the distribution of the incidence of breast cancer, which is known to be related to oestrogen levels. Even after all other risk factors for breast cancer have been taken into account, the incidence among Japanese women remains inexplicably low. Perhaps this puzzle, the so-called 'Japanese factor' of breast cancer¹², is related to low double-ovulation frequencies and low hormone levels.

Short's intriguing study¹ raises more questions than it solves. The nature of the causal links, if any, between testis size,

dizygotic twins and breast cancer remains to be clarified. Another study shows no relation between dizygotic twinning and breast cancer¹³. The primary observations of variation need extension — for example, do Yorubas have large testes as well as frequent dizygotic twins? It remains unknown whether differences in testis size arise from differences in tubule number, diameter or length, or in interstitial cells. What is clear from Short's work is that variation in human sex organs and function warrants serious study. □

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Astrophysics

Galaxy distances and deviations from universal expansion

from J. Richard Bond and Sidney van den Bergh

Two major findings involving large-scale structure in the Universe dominated discussion at a recent meeting*. The first of these was the result of the deep (out to $\sim 150 h^{-1}$ Mpc) extension of the Harvard/Smithsonian Center for Astrophysics redshift survey¹ that most galaxies and clusters in the Universe appear to lie on the surfaces and along the intersections of huge bubble-like structures (Hubble bubbles) enclosing voids with typical diameters $\sim 25 h^{-1}$ Mpc, where h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$. This work, which was discussed last month in these columns², confirms the picture reviewed by Oort in 1983 that superclusters, filaments and voids dominate structure on the largest scales³. A graphic example of the sort of structure which now seems pervasive is provided by the Perseus-Pisces supercluster, discussed at the workshop by M. Haynes (Arecibo Observatory). It is characterized by large-scale filamentariness stretching from the Perseus to the Pisces clusters, accompanied by holes in the apparently evacuated surroundings. N. Bahcall (Space Telescope Science Institute) presented a strong case that Abell clusters have the remarkably large correlation length $\sim 25 h^{-1}$ Mpc; Bahcall also suggested that superclusters defined by associations of rich clusters have an even larger correlation length. B. Tully (Hawaii) showed tentative evidence from the distribution of rich clusters that an extremely large ($\sim 300 h^{-1}$ Mpc) flattened structure exists of which the Local Supercluster is a small part.

The second major discovery reported at the meeting was that regions of the Universe with dimensions $\geq 50 h^{-1}$ Mpc are streaming with velocities of many hun-

dreds of kilometres per second relative to the absolute standard of rest provided by the cosmic microwave background (CMB). Previously it had been thought that the $610 \pm 50 \text{ km s}^{-1}$ motion of the Local Group relative to the microwave background resulted from a superposition of Local Group infall into the Virgo cluster and infall of the Virgo cluster into the Hydra-Centaurus supercluster. It now turns out that the Hydra-Centaurus supercluster was something of a red herring — Infrared Astronomical Satellite (IRAS) observations show that this complex does not have a massive extension hiding behind the dusty clouds of the Southern Milky Way. To cause the observed motion of the Local Supercluster would require the Hydra-Centaurus supercluster to have ≥ 5 times as large a mass-to-light ratio as the Virgo cluster. Furthermore the attraction by Hydra-Centaurus is partially offset by the Perseus-Pisces supercluster that is pulling us in the opposite direction.

New techniques, developed independently by G. Djorgovski (Harvard) and by D. Burstein (representing a large international group), make it possible to determine the absolute magnitudes of elliptical galaxies with an accuracy of ~ 0.5 mag (apart from an arbitrary zero point) and hence the distances to clusters to an accuracy of 5–10 per cent. Using this distance indicator, Burstein reported that Hydra-Centaurus is also moving with a velocity $\sim 700 \text{ km s}^{-1}$ relative to the microwave background as part of a relatively coherent streaming motion extending over at least $\sim 40 h^{-1}$ Mpc. Work on the original Rubin-Ford⁴ sample of spiral galaxies, extending from $\sim 35 h^{-1}$ Mpc to $\sim 65 h^{-1}$ Mpc, reported by C. Collins, R. Joseph and N. Robertson (Imperial Col-

- Short, R.V. in *One Medicine* (eds Ryder, O.A. & Byrd, M.L.) 32 (Springer, Berlin, 1984).
- Hayward, P. & Shire, J.G.M. *Nature* **250**, 499 (1974).
- Short, R.V. in *Reproductive Biology of the Great Apes* (ed. Graham, C.E.) 319 (Academic, New York, 1981).
- Kim, D.H. & Lee, H.Y. *J. Korean med. Ass.* **25**, 135 (1982).
- Land, R.B. *Nature* **241**, 208 (1973).
- Islam, A.B.M. *et al. Genet. Res.* **27**, 23 (1976).
- Bulmer, M.G. *The Biology of Twinning in Man* (Clarendon, Oxford, 1970).
- Short, R.V. *Ciba Fdn Ser.* **64**, 377 (1979).
- Nylander, P.P.S. *J. Obstet. Gynaec. Br. Commonw.* **80**, 651 (1973).
- Nylander, P.P.S. in *Twin Research: Biology and Epidemiology* (eds Nance, W.E. *et al.*) 35 (Liss, New York, 1978).
- Soma, H. *et al. Obstet. Gynaec., N.Y.* **46**, 311 (1975).
- Pike, M.C. *et al. in Banbury Report No. 8, Hormones and Breast Cancer* (eds Pike, M.C. *et al.*) 3 (Cold Spring Harbor Laboratory, New York, 1981).
- Wyshak, G. *et al. J. Natn. Cancer Inst.* **70**, 593 (1983).

*NATO Workshop held at Kona, Hawaii, 13–17 January 1986.

lege), and discussed on page 506 of this issue⁵, indicates streaming velocities averaged over the shell of $970 \pm 300 \text{ km s}^{-1}$ in the CMB rest frame. A sample of spirals associated with clusters used by M. Aaronson, J. Huchra and J. Mould using the Tully–Fisher distance indicator seem to infer slightly lower streaming velocities than the Burstein ellipticals which are, however, still many hundreds of kilometres per second. On the other hand, A. Meiksen (Berkeley) and A. Yahil (Stony Brook) showed that the IRAS galaxies have a dipole approximately in agreement with the direction of the microwave background dipole, reassuring us that the rest frame of the galaxies in a sufficiently large volume may eventually coincide with the CMB rest frame.

Models

These observational results severely challenge the currently popular theories of cosmic structure formation, and it is not clear whether any model universes so far proposed can satisfy all of these new large-scale results while generating the small-scale structure necessary to explain galaxies. Universes dominated by cold dark matter with adiabatic fluctuations of the form predicted in inflationary cosmologies have been relatively successful in explaining the correlation function and pair velocities of galaxies, as many of the theorists at the meeting emphasized^{6–10}. If bright galaxies are assumed to form mainly at high peaks of the mass density field (biased galaxy formulation), then the theory offers the hope of reconciling how the density parameter Ω could be unity despite the indications to the contrary from virial velocities. These positive features require that the Universe be dynamically young on the scale of rich clusters and beyond. Although it seems possible that the Hubble bubbles can be explained as pancakes just turning around along the line of sight, and the rich cluster abundances and overall correlation amplitude on scales $\leq 25 h^{-1} \text{ Mpc}$ more or less work out, there are serious problems with the shape of the rich cluster correlation function reported by Bahcall, and there is apparently no way the large streaming velocities unveiled at the meeting can be explained with this model. Unfortunately, the $\sim 600 \text{ km s}^{-1}$ streaming motions imply a Universe which is dynamically old on cluster scales, and it is hard to understand why galaxies avoid the huge ($\geq 10^{16} M_{\odot}$) clusters where most of the dark mass of the Universe would reside. The streaming motions suggest mass is more clustered than the light, precisely opposite to the biased galaxy formation assumption.

J. Ostriker (Princeton) attempted to explain the Hubble bubbles as natural consequences of the evolution of large-scale underdense regions of positive gravitational potential energy, which evacuate

the interior mass into dense shells (essentially pancakes) forming at their expanding boundaries. The largest bubbles would arise through merging of smaller-scale bubbles into ‘superbubbles’ in much the same way as shock waves add. The underdense initial bubbles could arise via the injection of explosive energy from supernova, for example, or be present in the initial conditions associated with primordial density fluctuations. It is not clear how the large streaming velocities can arise in such models for the same reason as in the cold dark matter case, and incidentally, in massive neutrino-dominated universes. Further, Bahcall’s clustering of clusters is also difficult to explain in this model.

Perhaps the most promising candidate theory, reviewed by J. Primack (Santa Cruz) and which will be discussed next week in these columns, is one in which cosmic strings formed at a phase transition in the very early Universe. Matter responds to the gravitational potential generated by the strings ultimately to condense into galaxies and rich clusters. Large-scale correlations, such as those rich clusters apparently have, appear naturally in such theories¹¹, associated with the matter response to large-scale loops of cosmic string. Whether the string theory is the universal panacea for what ails the current theoretical picture will only be decided when the predictions of this theory are made more quantitative. In particular, the large-scale streaming motions have not really been explored. Armed with the results of this meeting, theorists will be kept busy trying to determine more quantitatively which model universes can be excluded. Preliminary indications certainly suggest that viable universes with high velocities coherent over large regions are going to prove difficult to construct. The popular cold dark matter picture with biased galaxy formation may well be the first victim.

Hubble constant

Despite the surprising large-scale deviations from the Hubble flow reported at this meeting, a well-defined global Hubble parameter H can still be found, using brightest cluster members, as A. Sandage emphasized. However, the value still remains uncertain by almost a factor of two. Sandage and G. Tammann maintained that $H \approx 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$, whereas G. de Vaucouleurs claimed that $H \approx 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, and Aaronson and Visvanathan reported intermediate values.

There is now general agreement that the uncertainty in the Hubble constant is not caused by errors in the distances of Local Group galaxies. Feast showed that all distance estimates for the Large Magellanic Cloud (except perhaps those based on cluster main-sequence fitting) are now consistent with a distance of 50 kpc. The recent discovery of RR Lyrae stars in the

halo of the galaxy M31 by S. Pritchett and C. van den Bergh also fixes the M31 distance to be close to 700 kpc, in good agreement with previous determinations using Cepheids¹², novae¹³ and giant stars of Population II (ref. 14). Finally Aaronson reported on the discovery of two 30-day Cepheids in M101 that give a distance closer to that expected by Sandage and Tammann than by de Vaucouleurs, although absorption within M101 itself must be better determined to finalize this. Planned observations of novae in Virgo should considerably reduce the remaining uncertainty in the Hubble parameter.

The age of the Universe derived from the Hubble constant must be compatible with those derived using other methods (provided that the cosmological constant is zero). A. Renzini showed that all recent investigations of the age of globular clusters still give values in the range $(15–18) \times 10^9 \text{ yr}$. These values could be decreased by $\sim 3 \times 10^9 \text{ yr}$ if the CNO group elements are significantly less depleted in metal-poor objects than are iron and other well-observed heavy elements; certainly oxygen is relatively more abundant than iron in Population II objects than in Population I. A review by F. Thielemann showed that the age of the Galaxy determined by nuclear cosmochronology was only constrained to lie in the range $(11.6–24.6) \times 10^9 \text{ yr}$ as a result of uncertainties in determining the production ratios of the main chronometers $^{235}\text{U}/^{238}\text{U}$, $^{232}\text{Th}/^{238}\text{U}$ and $^{187}\text{Re}/^{187}\text{Os}$ and the time-dependence of r -process nucleosynthesis during Galactic evolution. W.A. Fowler maintains that a value closer to the lower end, $\sim 12 \times 10^9 \text{ yr}$, is preferred. In any case, the globular cluster and cosmochronology ages are barely consistent with an $H = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, $\Omega = 1$ cosmology, and are certainly incompatible with an $H = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ Friedmann universe unless a cosmological constant is included. $\square$

1. de Lapparent, V., Geller, M. & Huchra, J. *Harvard-Smithsonian Center for Astrophysics Preprint No. 2231* (1985).
2. Silk, J. *Nature News and Views* **320**, 12 (1986).
3. Oort, J. A. *Rev. Astr. Astrophys.* **21**, 373 (1983).
4. Rubin, V.C., Ford, W.K. & Rubin, J.S. *Astrophys. J. Lett.* **183**, L111 (1973).
5. Collins, C.A., Joseph, R.D. & Robertson, N.A. *et al. Nature* **320**, 506 (1986).
6. Blumenthal, G.R., Faber, S.M., Primack, J.R. & Rees, M.J. *Nature* **311**, 517 (1984).
7. Kaiser, N. in *Proc. Inner Space/Outer Space Conf.* (eds Kolb, E.W. *et al.*) (University of Chicago Press, 1985).
8. Davis, M., Efstathiou, G., Frenk, C. & White, S.D.M. *Astrophys. J.* **292**, 371 (1985).
9. Bardeen, J.M., Bond, J.R., Kaiser, N. & Szalay, A.S. *Astrophys. J.* (in the press).
10. Deckel, A. & Silk, J. *Astrophys. J.* (in the press).
11. Turok, N. *Phys. Rev. Lett.* **55**, 1801 (1985).
12. Welch, D.L., McLaren, C.W., McLaren, R.A. & Madore, B.F. *Astrophys. J.* (in the press).
13. Cohen, J.G. *Astrophys. J.* **292**, 90 (1985).
14. Mould, J. & Kristian, J. Preprint (1985).

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Confederations and human ecology

SIR—It was argued in *Nature's* Opinion columns¹ that Europe has yet to get its act together as a genuinely integrated unit. And much is currently made of the expected efficacy of confederation. But does confederation represent natural progress or is it only a transient cultural fashion and are exhortations to throw off our xenophobic tendencies properly founded or should we forever be distrustful of unification? Ecology may provide some of the answers.

The $-3/2$ law of plant distribution² has been shown to apply to animals as well as plants³. Not only can the natural relationship between mean size and distribution density of birds⁴, small mammals⁵ and primates in general⁶ be described by this theoretically substantiated⁷ allometric law but human distributions on the scale of nations also conform to it⁸. What does it have to say about confederations?

The $-3/2$ law can be portrayed as:

$$\log M = \log C - \frac{3}{2} \log \frac{N}{A} \quad (1)$$

in which M is the mean mass of a typical member of a community, C is a constant relating to the energetics constraints of the species and physical environment, N is the number of individuals and A is the area of their habitat.

Therefore, from (1):

$$\log M = \log C - \frac{3}{2} \log N + \frac{3}{2} \log A$$

so

$$\log NM = \log C - \frac{1}{2} \log N + \frac{3}{2} \log A$$

$$= -\frac{1}{2} \log N + (\log C + \frac{3}{2} \log A) \quad (2)$$

But NM is the total mass of the community, say M_T . Therefore, for a group occupying a fixed area so that A is constant, we have from (2):

$$\log M_T = -\frac{1}{2} \log N + \log B$$

where B is the constant $CA^{3/2}$. Hence $M_T = BN^{-1/2}$ (3)

Equation (3) describes a situation in which M_T increases as N decreases in a given constant area of unaltering physical environment occupied by a group of biological entities subject to fixed energetics characteristics. This is the case for nations of humans subject to parallel cultural advances as is essentially the case in modern Europe⁸. It is seen from equation (3) that, in order for the total mass (population) of a group of nations to expand, the number of independent nations must decrease. This implies the relinquishing of national boundaries to reduce wasteful competition in an ecological as well as economic sense.

The equivalent behaviour in plant communities involves the decay of one entity which imparts a resource to its neighbour in place of competition. In the

case of international confederations the only decay that is required is of parochial xenophobia, the sacrifice of national identity to the more successful growth of the greater community. Alternatively, especially in the global village, individual nations can remain aloof and await the rewards of the next technological revolution in order to relieve their energetics constraints, in an ecological sense, and thereby continue to grow albeit at a rate inhibited by comparison with their more co-operative neighbours.

Generally speaking history appears to have eventually favoured the less-efficient but perhaps more individually satisfying second option. Whether history or ecology will have the greater influence on the future remains to be seen.

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1. *Nature* **319**, 249-250 (1986).
2. Yoda, K., Kira, T.J., Ogawa, H. & Hozumi, K. *J. Biol. (Osaka)* **14**, 107 (1963).
3. Denness, B. *Proc. int. Conf. Is the Earth a Living Organism?* (National Audubon Society Expedition Institute, Sharon, Connecticut, 1986).
4. Schoener, T.W. *Ecology* **49**, 123-141 (1968).
5. McNab, B.K. *Am. Nat.* **97**, 133-140 (1963).
6. Clutton-Brock, T.H. & Harvey, P.J. *Zool.* **183**, 1-39 (1977).
7. Whittington, R. *Nature* **311**, 217 (1984).
8. Hayton, A.F. *Nature* **310**, 178 (1984).

The mesoscale effects of nuclear winter

SIR—The recent paper by Golding and colleagues¹ and accompanying *News and Views* commentary by Emanuel² appear to represent an awakening of interest among mesoscale meteorologists in the problem of 'nuclear winter'. From Emanuel's characterization of the calculation of Golding *et al.* as "a welcome step in transforming nuclear winter research from a means of political advocacy to a scientific exercise", readers might well conclude that removal of nuclear war-generated aerosols by mesoscale processes has been ignored in previous studies, presumably rendering the results of these studies "unscientific". Further, it is rare that scientists of high calibre characterize the research of their colleagues — even controversial work — in terms as harsh as it "has become notorious for its lack of scientific integrity". We are moved to respond since we have published several contributions on "nuclear winter", including several refereed pieces in *Nature*³⁻⁷ and, in the process, have tried to refrain from political advocacy.

The Golding *et al.* calculation is welcome since it is the only published mesoscale model applied to nuclear smoke that we know of, although it does not go nearly far enough to alter any possible conclusions connected with nuclear winter. For example, it has been well known and well

stated in the major nuclear winter assessments^{8,9} that early cloud-scale removal could be significant and was not explicitly included in any of the global circulation climatic models, whose grid scale resolution ranges from 500 to 1,000 km. Indeed, the National Academy of Sciences assumed that some 50% of initially produced smoke would be washed out in cumulus scale convection, and cloud-scale calculations by William Cotton of Colorado State University¹⁰ have already shown that initial intense heating of the surface from urban mass fires is very likely to generate large-scale convective activity. None of this is ignored in the bulk of the nuclear winter literature that we are aware of, and it is spelled out explicitly in all of our papers.

For example, in our first paper we specifically state that "the way fires will burn (for example, fire storms), the height to which smoke is injected, the duration of fires, the particle concentration within initial smoke plumes, and early particle removal by rainout in convective/mesoscale circulations all occur on spatial scales smaller than the resolution of any general circulation model now available".

Moreover, in a civil exchange with S.F. Singer in the pages of *Nature*^{11,12}, we conclude our discussion of Singer's criticisms with the following comment: "Mesoscale effects will be important in determining the removal of smoke from the atmosphere. Intuitively, one might expect enhanced moist convective activity around smoke clouds to increase the removal rate of particles, but it is not yet possible to explicitly calculate the effects of dense smoke clouds on mesoscale circulations."

Again, both in a semi-popular article⁵ and in the pages of *Nature*⁶, two of us have individually mentioned the uncertainties in applying the theory of nuclear winter on a local scale because of the mesoscale problem. Although the British Meteorological Office calculation is a step in the right direction, it does not include the most controversial and important aspects in the entire early removal debate: how individual smoke particles from multiple smoke plumes will be processed as they pass through fire-associated cumulus clouds and then through secondary mesoscale circulations, and the fraction of the initially generated smoke that will not be processed by such convective activities. Indeed, this parameterization of microphysics is just as much a problem in cloud scale, mesoscale and general circulation models, and is, in our opinion, the principal uncertainty in determining whether early removal will prove to be a 90% or a 10% issue. The US National Academy Report⁸ suggested early removal of 50% (on arbitrary grounds), and thus we still await further work.

In summary, we are content that our

contribution to the "recent literature on nuclear winter research" has been quite explicit in stating the limitations of our own work, and we specifically call for others to begin answering difficult questions at scales that will be perpetually sub-grid scale to most atmospheric models. Therefore we do not take the new calculation¹ as a step in moving nuclear winter "toward a scientific exercise" as characterized by Emanuel, but a logical progression of scientific research as already called for by most responsible researchers. This call was either unknown to or ignored by Emanuel in his *News and Views* comment.

We believe we have set the record straight, and hope that this effort will reinforce the notion that good scientific enterprise should be based upon sound empirical evidence, and that when such evidence does not exist appropriate caveats must accompany all statements.

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1. Golding, B.W., Goldsmith, P., Machin, N.A. & Slingo, A. *Nature* **319**, 301-303 (1986).
2. Emanuel, K.A. *Nature* **319**, 259 (1986).
3. Covey, C., Schneider, S.H. & Thompson, S.L. *Nature* **308**, 21-25 (1984).
4. Covey, C., Thompson, S.L. & Schneider, S.H. *J. geophys. Res.* **90**, 5615-5628 (1985).
5. Schneider, S.H. 1985 *Britannica Book of the Year*, 26-33 (Encyclopaedia Britannica, Chicago, 1985).
6. Thompson, S.L. *Nature* **317**, 35-39 (1985).
7. Thompson, S.L. *et al. Ambio* **XIII**, 236-243 (1984).
8. National Research Council Committee on Atmospheric Effects of Nuclear Explosions, *The Effects on the Atmosphere of a Major Nuclear Exchange* (National Academy Press, Washington, DC, 1985).
9. Pittcock, A.B., Ackerman, T.P., Crutzen, P.J., MacCracken, M.C. & Shapiro, C.S. *Environmental Consequences of Nuclear War*, SCOPE 28, Vol. 1 (Wiley, Chichester, 1986).
10. Cotton, W.R. *Am. Scientist* **73**, 275-280 (1985).
11. Singer, S.F. *Nature* **310**, 625 (1984).
12. Thompson, S.L., Schneider, S.H. & Covey, C. *Nature* **310**, 625-626 (1984).

Evolution with a Japanese slant

SIR—I neither contest your criticism¹ of the recent move in Japan to establish a research institute for Japan studies, which the Prime Minister Nakasone supports in order to "export" Japan and its pragmatic socioeconomic system, nor do I condone the role played by some established scientists in Japan in support of that programme.

But I would like to point out that the argument advanced in *Nature* by Dr Beverly Halstead² to refute the evolutionary theory of the Japanese scientist Imanishi needs scientific scrutiny. The references

that Halstead gives (see refs 3-8) do not substantiate the claim that the anti-darwinian theory of Imanishi "would not last long if subject to international attention".

Attempts by some Japanese scientists to underpin the current intellectual trends in Japan, which exalt traditional Japanese values, encompass not only "individualist" thinkers of Imanishi's "Kyoto Elite" (see ref. 1), but also those who base their arguments on developments in biophysics and information science^{9,10}. I have not spared criticism¹¹ of these ethnocentric excesses. Incidentally, Imanishi does not think the term "individualism" in the Western sense should be applied to himself; his view is that the individual can be "poetically" unified with nature without any self-assertion, though he concedes that his scientific career and endeavours would be classed as individualistic by Western thinkers.

On the main theme of Imanishi's evolutionary theory based on the lifestyle partitioning of mayfly larvae, Halstead argues (see refs 3-5) that the association of larvae of a given species is not a consequence of some type of proto-identity, as Imanishi conceives, but simply of a preference for particular physical parameters of the environment. More than a few Japanese biologists feel that, although Halstead has admirably succeeded in giving a good summary of Imanishi's theory, one would need, in order to make dialogues between east and west scientifically meaningful, to read the works of Imanishi¹² and Tokichi Kani¹³ (who was killed during the Pacific War, having been assigned to a hopeless battlefield, on account of his leftist ideology). These classics should be translated into English.

The crux of the matter is the *interaction* between different species, or species-societies as Imanishi puts it. Species will indeed have preferences for certain physical factors, but such preferences may well partially overlap, and thus are unlikely to explain the almost total segregation of habitat between allied species of mayflies observed by Imanishi. Of the five references Halstead gives in support of his thesis, three³⁻⁵ deal only with higher taxonomic groups, ignoring interaction between constituent species, and thus are irrelevant. The fourth⁴ describes experiments to characterize physical preference of burrowing larvae of only a single species of mayfly, and so is also irrelevant. Moreover, the last, dealing with temporal segregation of two trichopteran species at the same aquatic habitat⁷, actually serves as a good example of Imanishi's concept of lifestyle partitioning (*sumiwake*).

Halstead then goes on to state that recent researches "in experimental ecology have demonstrated⁶ that interspecific competition takes place in some 90 per cent of all cases studied". But what has

been demonstrated is merely the presence of *interaction* between species; this may be interpreted as resulting from competition, but Imanishi would argue otherwise. So criticism in terms of conventional concepts is unfruitful. Much more discriminating and extensive observations and/or experiments are needed to settle the question.

I should add that recent work by Kazumi Tanida¹⁴ on trichopteran or caddis-fly larvae of the genus *Hydropsyche* has confirmed Imanishi's earlier results on mayflies as well as those of Kani on various insects in freshwater streams. Tanida has found that each of the six species studied, at the very streams near Kyoto where Imanishi and Kani operated nearly half a century ago, occupies its own distinctive macro/microhabitat, sometimes exactly contiguous and non-overlapping with those of other species, and separate either seasonally, topographically or locally.

The problem is how such a pattern of lifestyle partitioning could have evolved. Only *ad hoc* hypotheses can explain it on orthodox neo-darwinian theory, but this is not so with the theory based on Imanishi's "proto-identity" concept. Naturally, there is a need to devise experimental tests of Imanishi's theory, which is admittedly somewhat unrefined and crude.

Andrew Rossiter, a young British freshwater entomologist, who advised Halstead in Kyoto in 1984 of those references³⁻⁸, has now returned to Japan for a substantial period, and is now apparently recognizing something significant in the work of Tanida, with whom he is collaborating. If Halstead had come to Japan a year later, he might have received different advice from Rossiter. Developments in the next few years will be awaited with interest.

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1. *Nature* **317**, 463 (1985).
2. Halstead, B. *Nature* **317**, 587 (1985).
3. Barber, E.W. & Keven, N.R. *Hydrobiologia* **43** (1-2), 53-75 (1973).
4. Hawkins, C.P. & Sedell, J.R. *Ecology* **62**, 387-397 (1981).
5. Scullion, J., Parish, C.A., Morgan, N. & Edwards, R.W. *Freshwat. Biol.* **12**, 579-595 (1982).
6. Wright, L.L. & Mattice, J.S. *Aquatic Insects* **3**, 13-24 (1981).
7. Cummins, K.W. *Ecol. Monogr.* **34**, 271-295 (1964).
8. Schoener, T.W. *Am. Nat.* **122**, 240-285 (1983).
9. Shimizu, H. in *Hyumansaiensu (Human Science)* Vol. 1 (eds Ishii, T., Kobayashi, N., Shimizu, H. & Murakami, R.) 29-80 (Nakayama Shoten, Tokyo, 1984). (In Japanese).
10. Ishii, T. *Horonikku Pasu (Holonic Path)* (Kodan Sha, Tokyo, 1985). (In Japanese).
11. Sibatani, A. *J. social biol. Struct.* **6**, 335-343 (1983); *Kurasis (Crisis) (Tokyo)* (Special issue on ecology, feminism and socialism) 143-153. (In Japanese).
12. Imanishi, K. *Seibutsu no Sekai (The World of Living Things)* (Kobundo, Tokyo, 1941); *Seibutsu Shakai no ronri (The Logic of Organic Society)* (Mainichi Shimbun Sha, Tokyo, 1949). (In Japanese).
13. Kani, T. in *Nippon Seibutsu Shi, Konchu 1. (Fauna and Habitats of Japan, Insects I)* (ed. Furukawa, H.) (Kenkyu Sha, Tokyo, 1944). (In Japanese).
14. Tanida, K. *Physiol. Ecol. Jap.* **21**, 115-130 (1984).

A word to the wise

Philip T. Smith

Knowledge of Language: Its Nature, Origin, and Use. By Noam Chomsky.
Praeger: 1986. Pp.307. Hbk \$29.95, £27.50; pbk \$9.95, £9.95.

ARE languages real? This may seem an unnecessary question to the owner of the *Oxford English Dictionary*, to the librarian, to the court reporter or to the man who has imprudently ignored a sign reading "Beware of the Dog". But there are unresolved issues about the status of a language that are remarkably difficult to deal with. The linguist Martin Joos put the matter well when he distinguished "hocus pocus" and "God's truth" approaches to language: *hocus pocus* linguists are looking for patterns in a language, but these patterns have no privileged philosophical or psychological status and any other set of patterns that fits the data is equally valid; God's truth linguists believe that there is a unique structure waiting to be discovered.

Up to a point these are problems common to all scientific endeavours: a physicist may worry about the extent to which atoms are "really" like billiard balls, or a chemist about the status of a concept such as valency. For the physicist or the chemist, however, direct observations of the world around them constrain their theorizing: the concepts of the billiard ball atom or of valency will be accepted, modified or discarded according to how well they contribute to successful theories of physical phenomena. With language things are more complicated because there is no general agreement as to what observations are relevant to our theorizing. Is it the external manifestations of a language, the totality of utterances of a particular speech community, that should form the basis of our testing of linguistic theories? Or is it the less-tangible internal knowledge of a language possessed by an individual that should be the starting point? Tradition, convenience and common sense say we should be concerned with the external manifestations of language: it is Noam Chomsky's most important contribution to linguistics that he equates the goals of linguistics with the study of internal knowledge.

Chomsky has always thrived on controversy. His contempt for behaviourism, reflected in his 1959 review of B.F. Skinner's book *Verbal Behavior*, has been extended to most of experimental psychology, which he believes to be merely the pointless gathering of data. He thrives, too, on the paradoxical statement (for example his claim that English orthography is "near optimal" would amaze the millions of people who have had trouble

learning to read English and to spell it correctly). But perhaps the greatest paradox of Chomsky's work is that his theory of language is based on the need to explain how almost any child can learn its mother tongue with relative ease, at the same time using as data for his theory only the intuitions of a small number of highly educated adults like himself.

Chomsky's central argument runs as follows. We need to move away from the conception of language as an external object (an "E-language") because studying such a language is merely an exercise in discovering patterns in the data, without being able to explain why such patterns are there and without being able to choose between alternative competing descriptions of the data. Instead, argues Chomsky, we must conceive of language as an internal state of an individual's knowledge (an "I-language"). The goal of linguistic theory is to describe this knowledge in terms of a universal grammar (UG) which is common to all human languages and to all human beings. Universal grammar is not totally inflexible, otherwise we would all speak the same language; rather, UG contains certain parameters that differ between languages. The child's goal is to discover what values these parameters take for the language being presented to him; the linguist's goal is to discover the form of UG and to write grammars for particular languages which incorporate the principles laid down in UG. Simultaneously, this argument sketches the solution to two very difficult problems. First,

how can a child learn a language so quickly on so little evidence? (answer: the number of possible grammars the child has to consider is radically reduced by UG, and UG is part of the child's innate endowment). Second, how can a linguist choose between competing descriptively adequate grammars for a single language? (answer: choose the grammar that is most in harmony with the principles of UG). Thus when linguists sit in their offices in MIT arguing about the appropriate analysis of an English sentence such as "John is too stubborn to expect anyone to talk to", their concerns are similar to those of a naked child living in a community whose language is spoken by a few hundred people in the depths of Papua New Guinea. Both need access to UG to succeed.

In this, his latest book, Chomsky has restated his views on the nature of language, and linguists who have kept in touch with his work in recent years will find little that is new to them. Rather, the main achievement of the book is the long central chapter which sets out with great lucidity the development of ideas about the nature of language ("generative grammar") over the past 30 years. Particularly noticeable is the shift from using many specialist rules to account for facts about English to an emphasis on general principles that are incorporated in UG. In a later chapter Chomsky deals at length with the notion of what it means to obey a rule, and counters objections from philosophers, principally Wittgenstein, Searle and Dummett.

Linguists and psychologists ignore Chomsky's work only at their peril, but I have one substantial reservation about his current theoretical position. This is the reliance on the notion of a "best" grammar: the child or the linguist is supposed to select the best among possibly several competing grammars, the criteria for "best" being incorporated in the theory of UG. Chomsky cannot yet spell out what

IMAGE
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REASONS

"Blocks world": a highly simplified domain which is used by workers in artificial intelligence to explore the ways that perception, thought and actions (for example in stacking the shapes) can be modelled and linked by the computer. The illustration is taken from *Language, Writing, and the Computer: Readings from Scientific American* published by W.H. Freeman. Price is hbk \$20.95, £20.95; pbk \$12.95, £12.95.

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all these criteria will be, but it seems clear that he thinks that one of them is redundancy: for example, given two descriptively adequate grammars G and G' , we should prefer grammar G to grammar G' if G' contained "superfluous rules" without which G' would be "essentially equivalent to G " (p.250).

Now, from a biological perspective redundancy is not necessarily pernicious: duplication of information in an organism is a protection against temporary or permanent malfunction of one of its constituents, and a partially redundant grammar, containing repetitions and special cases that are "better" handled by general rules, may turn out to be biologically superior to the minimally redundant grammar favoured by the linguist. Moreover true insight into the form of UG might be possible only with this biological version (because, for example, certain constraints might be related to particular physiological properties of nerve cells, or, at a more psychological level, the capacity of human short-term memory might preclude rules of a particular degree of complexity). In some areas of theoretical psychology (for

example Marr's work on vision) it may be possible to separate a computational theory of why particular procedures should be performed from an algorithmic theory of how they are performed. This separation is possible because the properties of the real world form part of the justification for why certain procedures are necessary. Our visual systems may have evolved the way they are partly because objects tend to be rigid, show certain symmetries and so on. But there is no comparable external evolutionary pressure for UG to possess certain properties (this point is explicitly acknowledged by Chomsky, p.274, note 22). Only the constraints on the biological manifestation of UG are relevant, and this manifestation, I have suggested above, may be partially redundant and repetitious. This leaves the linguist's version of UG as an unmotivated abstraction, the very thing that Chomsky has been working against. The question is, is it only the child in the Papua New Guinean village who has no clothes? □

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On the paper chase

John Galloway

Elements of the Scientific Paper: A Step-by-Step Guide for Students and Professionals. By Michael J. Katz. *Yale University Press: 1986. Pp.130. Hbk \$25, £18; pbk \$8.50, £5.95.*

SCIENCE must now be one of the world's largest industries. What it manufactures, of course, is knowledge and like other goods this knowledge has to be sold. The way this is done is by the publication of the papers that make up the scientific literature. Unlike most industries, however, production and sales are the responsibility of the same person — the scientist; and when it comes to writing papers the average scientist does not do it all that well, the tendency at the moment being to write in the inert prose of a sort of machine code.

Scientists, then, are in need of all the help they can get. In response, a new industry has arisen, that of telling them how to write. Some thirty books can readily be found in this genre, to which we must now add yet another. Professor Katz takes us through one of his own papers — on axonal growth — section by section with tips along the way for avoiding some of the loose usage of English which is characteristic of the scientific paper. This is a worthy enough aim, although if you want to know how to use English, Fowler tells you more fully, Mark Twain more wittily.

Even so, it would be churlish not to wish

well an attempt to get scientists to be clearer in their writing. On the other hand, at the back of my own mind when writing papers was the aphorism "if your thoughts are rubbish merely, be certain not to write too clearly". From the evidence of the large proportion of papers that are cryptic when they are not incomprehensible, that feeling is not uncommon.

For a non-scientist the book would, I think, merely reinforce the notion that the acquisition of scientific knowledge and understanding is somewhat mechanical. I don't think that Sir Peter Medawar's "Is the Scientific Paper a Fraud", with its exhortation to scientists to tell the truth about their thought processes, had that much influence. For example, few people would get away with Francis Crick's "I first learned of it [the new idea] sitting round a campfire in Aspen, Colorado" in a paper in *Trends in Neurosciences*. I sat up, though, at Professor Katz's suggestion that any results that don't fit should be put in a drawer and forgotten; everyone does it, but it's supposed to be a secret.

Professor Katz also tells us that a paper should be like a detective novel and, elsewhere in the book, that it is like a poem (that is, with a well defined form). These are nice analogies. The poetical form should of course mimic the limerick which, as Clifton Fadiman said, possesses progression, development, variety, speed, climax — and a high mnemonic value. Above all a paper, like an advertisement, has to be remembered. □

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Textbook reprise — animal behaviour

Richard Cowie

Unravelling Animal Behaviour. By Marian Stamp Dawkins. Longman: 1985. Pp. 159. Pbk. £6.50, \$12.95.

Animal Behaviour: Psychobiology, Ethology and Evolution. By David McFarland. Longman/Benjamin-Cummings: 1985. Pp. 576. Pbk £11.95; hbk \$28.95.

An Introduction to Ethology. By P.J.B. Slater. Cambridge University Press: 1985. Pp. 195. Hbk £22.50, \$39.50; pbk £7.95, \$12.95.

Animal Behaviour: A Concise Introduction. By Mark Ridley. Blackwell Scientific: 1986. Pp. 150. Pbk £8.80. To be published in the United States by Mosby.

THE arrival on my desk of four new undergraduate textbooks on animal behaviour prompted me to find out how many such books are currently in print: there are 34, not including books for the layman or more specialized volumes on, say, behavioural ecology. Obviously, to have any impact a new book must cover fresh ground or else be clearly better than its competitors. Of these four, one falls into the former category, one into the latter, and two into neither.

The book which breaks new ground is Marian Dawkins's *Unravelling Animal Behaviour*. To be fair to the other authors, this does not set out to be a conventional textbook or review. It is, rather, a collection of ten essays tackling conceptual problems in animal behaviour which have led to confusion in the literature and, in some cases, to controversy. Thus we find chapters on adaptation, optimality, inclusive fitness, the genetic basis of behaviour, the nature-nuture controversy, animal communication and sexual selection. Without exception Dawkins writes clearly and, more importantly, thinks clearly about these topics. I approached the book with a certain air of superiority, convinced that I understood the issues involved despite the author's admission that she herself had stumbled over many of them. How wrong I was. Particularly enlightening are the essays on inclusive fitness, where Dawkins discusses the mistake of using a simple weighted sum to calculate the benefits of helping relatives, and that on evolutionary stable strategies, where she sorts out the confusion between mixed and conditional strategies. There are few new ideas in this book, yet it makes explicit what was previously often implicit.

David McFarland's *Animal Behaviour*

makes a substantial contribution to the behavioural literature. It provides us with a more modern version of Hinde's classic textbook of the same name. Like Hinde, McFarland's strength is in the integration of material from ethology and comparative psychology to give a very comprehensive treatment of the subject; unlike Hinde's book, however, it is easy to read and has the sort of supporting features that are standard in today's textbooks.

Each of the main sections of the book begins with a short historical profile of a scientist who made a major contribution in the particular field considered, and each chapter ends with a summary of salient points. Between them, the various sections cover everything one would expect from the title — genetics and behaviour, natural selection, evolution and social behaviour, animal perception, the animal and the environment, animal learning, instinct, decision-making in animals, and the mentality of animals. McFarland is at his best on the more psychological topics (the sections on learning and imprinting are especially good). More purely ethological subjects, such as communication and social behaviour, are dealt with less adequately, but surprisingly it is those areas in which McFarland has himself carried out research that are the weakest. This is because he tends to present his own and his students' work at the expense of that of others; for example, in the chapters on optimality and decision-making over 60 per cent of the references relate to work by McFarland and his co-workers. Whilst they have unarguably made an important contribution in this area, it has been overemphasized here. But this is a relatively minor criticism; overall *Animal Behaviour* is an excellent textbook.

The two remaining books, by Slater and by Ridley, are very similar. Both are introductory texts which are aimed, I would imagine, more at sixth-formers than undergraduates. Both are well written and enjoyable to read; both are well illustrated; and both tend to use the same classic (well-worn?) examples.

The title of Slater's book is the more honest as neither book contains much information about experimental psychology; Slater deals with learning in three pages and Ridley in two. In fact, Ridley has some rather surprising gaps: there is only a passing reference to motivation in one paragraph and little information on stimulus selectivity. In general he concentrates on functional explanations rather than causal ones. Slater's book is better balanced, being divided fairly equally between the two approaches. Slater's chapter on motivation is particularly good, and he explains clearly the developments in theory from Lorenz's psychohydraulic model to McFarland's "state space" approach.

The two authors have used different techniques to avoid cluttering the text with references. Ridley provides a brief review of sources at the end of each chapter, while Slater includes references in the figure legends but not the text. This latter strategy can prove irksome, however; if a particular example does not have a figure to accompany it, it is impossible for the reader to trace the paper concerned.

Ridley's book, too, has its irritations. First, his definition of behaviour as a



From *Animal Behaviour* by David McFarland

Following ethology: Konrad Lorenz trailed by imprinted goslings.

series of muscular contractions is too narrow, because it rules out colour changes which can play an important role in communication. Secondly, several of the figures are redundant: for example a whole page (Fig. 1.3) is devoted to a sequence of a gull swallowing a chick (which, incidentally, is not the black-headed gull referred to in the text). Finally, in trying to be concise the book tends to oversimplify, a pitfall that Slater manages to avoid.

Altogether, then, 1985 was a year in which students of animal behaviour were well served by the publishing industry. McFarland's book must rate as one of the best undergraduate textbooks on the subject currently on offer, while Dawkins's would be an excellent basis for a series of tutorials or class discussions. Slater and Ridley have both produced elementary accounts. The two are similar, although Slater provides a more even coverage of the subject. □

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• The annual textbook review supplement appeared in *Nature* of 27 February 1986.

The hard and lonely road to science

Brenda Maddox

Hypatia's Heritage: A History of Women in Science from Antiquity to the Late Nineteenth Century. By Margaret Alic. *The Women's Press, 124 Shoreditch High Street, London E1 6JE, UK: 1986. Pp. 230. Pbk £4.95. To be published in the United States by Beacon Press, Boston.*

THE belief that the intellectual woman's place is in the humanities dies hard. When women do enter science, they are widely expected to keep to the gentler side. As the Queen Mother once said when visiting a University of London college: "Surely only girls do botany!"

In *Hypatia's Heritage*, Margaret Alic musters a great deal of historical evidence to show that women have always been active in scientific thought and research. Ms Alic, a molecular biologist who has taught a course on women and science at Portland State College in Oregon, rejects entirely Freudian musings that men's scientific adventurousness may be an extension of their curiosity about their mother's body — in other words, that they are driven by a sense of mystery about the natural world which women do not have.

Her case, surprisingly, neither begins nor ends with Rosalind Franklin, who modern feminists have argued did not get fair recognition for her contribution to the discovery of the double helix. It begins instead in prehistory, when, according to Ms Alic, women as food-gatherers became versed in horticulture and agricultural science. It ends just before Marie Curie. After Madame Curie, the author believes, the structure of science changed, amateurs gave way to professionals, and qualified women could advance, although with difficulty.

Yet, in between, a great number of distinguished women scientists suffered from having others enjoy the fruits of their research. For centuries women's scientific work was published anonymously or under the name of a husband or colleague. The heroine for whom the book is entitled — Hypatia of Alexandria — was a fourth-century astronomer and mathematician who wrote widely on the development of algebra and Ptolemaic astronomy. Much of Hypatia's writing appeared under the name of Theon, who revised Euclid's *Elements of Geometry*. (Hypatia came to an unhappy end, drawn and quartered, not for her sex but for her refusal to convert to Christianity.)

According to Ms Alic, women scientists fared well in the Middle Ages. The cloisters and convents allowed them intellec-

tual freedom and some were rewarded with places on the faculties of the early universities: in the fifteenth century, at the age of 25, Maria di Novella became head of mathematics at the University of Bologna. The Reformation, in dissolving the convents, deprived women of this educational heritage, argues Ms Alic — and of the option of not marrying. For several centuries afterwards science became the province of the determined male amateur and women's participation was largely as "scientific ladies", running salons and looking over men's shoulders. In 1667 there was considerable debate at the Royal Society before Margaret Cavendish, the Duchess of Newcastle and author of 14 scientific books, was admitted simply for a visit. And, in the eighteenth century, Lady Mary Wortley Montagu was learned enough to introduce into Europe a type of immunization against smallpox, yet her advice on the education of her granddaughter was "to conceal whatever learning she attains, with as much solicitude as

she would hide crookedness or lameness; the parade of it can only serve to draw on her . . . envy . . . and hatred".

As a feminist book, *Hypatia's Heritage* is happily unpolemical but a tiny bit dull. It bears the stamp of the lecture notes from which it was drawn; example is piled upon example like brick upon brick. A touch of the Germaine Greers might have helped, but then a tone of crusading passion would have been inappropriate. The author does not seem to see enormous progress but rather that, at the end of thousands of years of history, women of science still face a hard and lonely road. It is still true, she concludes, borrowing the words written by Henrietta Bolton in *Popular Science Monthly* in 1898, that "as a general rule the scientific woman must be strong enough to stand alone, able to bear the often unjust sarcasm and dislike of men who are jealous of seeing what they consider their own field invaded". □

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Myth in the making

Derek Paddon

The Fifth Generation Computer: The Japanese Challenge. By Tohru Moto-oka and Masaru Kitsuregawa. Translated by F.D.R. Apps. Wiley:1985. Pp.122. Pbk £8.95, \$17.95.

IN under 30 years computer technology has spanned four generations: the electronic valve, transistors, large scale integrated circuits on silicon and very large scale integrated circuits (VLSI). The fifth will combine VLSI technology and artificial intelligence to give the most advanced and influential machine in the history of mankind. A project to build such a machine was announced by the Japanese in 1981; international response was swift and other national projects were quickly set up.

According to Professors Moto-oka and Kitsuregawa, the fifth-generation computer will be the leading computer in the 1990s. The areas of application of these machines will be vast — in offices, classrooms, factories and laboratories, for language understanding and translation, picture processing, making business decisions, government policy-making, and improving productivity in manufacturing and public services; in other words, they will aid us in all activities that require mental ability.

Moto-oka and Kitsuregawa have produced a very readable account of the aims and background of the fifth-generation project. The technical demands on the reader are low; certainly, non-specialists will find it readily comprehensible. Unfor-

tunately, however, the research aspects of the book are very misleading. If the fifth-generation computer is to become a reality, many fundamental research problems must be solved, ranging from its basis of "strong" artificial intelligence to the novel computer architecture it will require. The authors imply that incremental development will resolve these difficulties; that, quite simply, seems unlikely.

During the early stages, the view emanating from Japan was that the Institute for New Generation Computing (ICOT), located in Tokyo, was solely responsible for research, with hardware support coming from the Japanese electronics companies. The authors continue to convey this image. Those familiar with Japanese industry, however, regard ICOT as but the focal point of the research and not the mainstay of the national effort. If this is not so, we are supposed to believe that some 70 personnel will succeed where thousands of IBM engineers and scientists have not.

I regret, then, that the authors did not give an analysis of the fifth-generation project as seen three years on. Instead we again have to be satisfied with a contradictory account which leaves us asking the same questions that have remained unanswered since 1981. □

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Spring books

Next week's issue of *Nature* includes the Spring books supplement. Reviewed are the autobiographies of P.B. Medawar and Rudolf Peierls, a biography of Max Delbrück, and books by Julian Schwinger, Bernard d'Espagnat, Elliot Valenstein, Niles Eldredge, Linus Pauling, Harry W. Paul and John Maynard Smith.

Building damage in Mexico City earthquake

from Adrian M. Chandler

One lesson to be learned from the recent Mexico City earthquake is that the Mexican earthquake-code torsional design recommendations for asymmetric buildings do not include a sufficient safety margin.

To engineers, the most disturbing aspect of the recent earthquake disaster in Mexico was the type and extent of the buildings which failed. Many were modern, engineered structures designed and built to withstand just such an occurrence. In the central area of Mexico City, 177 buildings collapsed completely and 85 suffered partial collapse as a result of the magnitude (M) 8.1 earthquake of 19 September 1985 and its aftershocks¹, while virtually every building in the city suffered some form of foundation failure. The nature and severity of this devastating earthquake has already forced a major reappraisal of engineering and architectural practices in earthquake-prone areas of the world.

Mexico itself has one of the most advanced building codes for earthquake-resistant design², but the extent of its enforcement and possible corruption in construction practices has come under sharp attack in the wake of the disaster³. The unprecedented severity of the earthquake, however, was chiefly responsible for the extent of the damage caused.

A joint venture by the US National Science Foundation and the Instituto de Ingenieria de la Universidad Nacional Autonoma de Mexico funded the installation only weeks before the earthquake of an array of 20 state-of-the-art accelerographs spread along the west coast of Mexico north of Acapulco. The accelerograph project was set up to record the strong-motion earthquakes predicted for the Guerrero seismic gap in the Mid-American trench. Major ground move-

ments were expected in the area because there had been a 75-year lull following a decade (1900–1911) in which 24 large earthquakes had occurred. In addition, a network of accelerographs had been set up over the past 30 years in and around the capital city. Thus the documentation collected from the Mexico City earthquake and aftershocks constitutes the most detailed and extensive ever recorded, and full analysis of the mass of information generated will take several months or even years. Work so far has concentrated on the initial earthquake. Careful analysis is being made of the source of the shocks beneath the Pacific near Playa Azul. The first evidence from the coastal arrays is that the earthquake contained a significant proportion of its energy at frequencies of ~ 0.5 Hz. Examination of data from the arrays established farther inland will show how the initial spectrum of vibrations was attenuated or amplified as it spread out across the country. Of interest to engineers is the local amplification of ground motion imparted by Mexico City's soft lake-bed subsoil which resulted in a series of 0.5-Hz shockwaves at ~ 400 km from the earthquake epicentre.

Resonance of Mexico City's subsoil at a period of ~ 2 s was observed during the earthquake on 28 July 1957 (ref. 4), but its significance was not fully appreciated at that time. Far less damage occurred in the city than during the latest earthquake, but the event prompted the establishment of a building code for seismic design⁵ (one of the first of its kind), together with the foundation of the engineering institute.

Engineers in Japan and the United States in particular are awaiting the results of seismological surveys which will analyse to what extent the apparent local amplification of the 2-s ground waves is peculiar to the sedimentary basin of Lake Texcoco on which Mexico City is founded (Fig. 1a); there may well be important implications for places such as Oakland in California and the many Japanese cities that are built on soft alluvial coastal plains⁶. Both the Californian Earthquake Engineering Research Institute (EERI) and Britain's Earthquake Engineering Field Investigation Team (EEFIT) have sent groups of engineers to study the damaged areas and to report on why some buildings survived while others were completely destroyed. Visits of this nature are now commonplace following major earthquakes. EEFIT, for example, is a UK-based group of engineers and scientists with considerable experience in post-earthquake reconnaissance in Italy, Turkey, Chile, North Yemen, Belgium and Pakistan. A preliminary report on the Chilean earthquake ($M = 7.4$) of 3 March 1985⁷ cited topographical effects similar to those in the region of Mexico City as a likely cause of the concentration of earthquake energy into the particular part of the alluvial plain on which the damaged towns are situated.

Comparison with Europe

Long-period amplification of ground motions has also been observed and extensively reported in eastern European earthquakes^{8–11}. The Vrancea region of

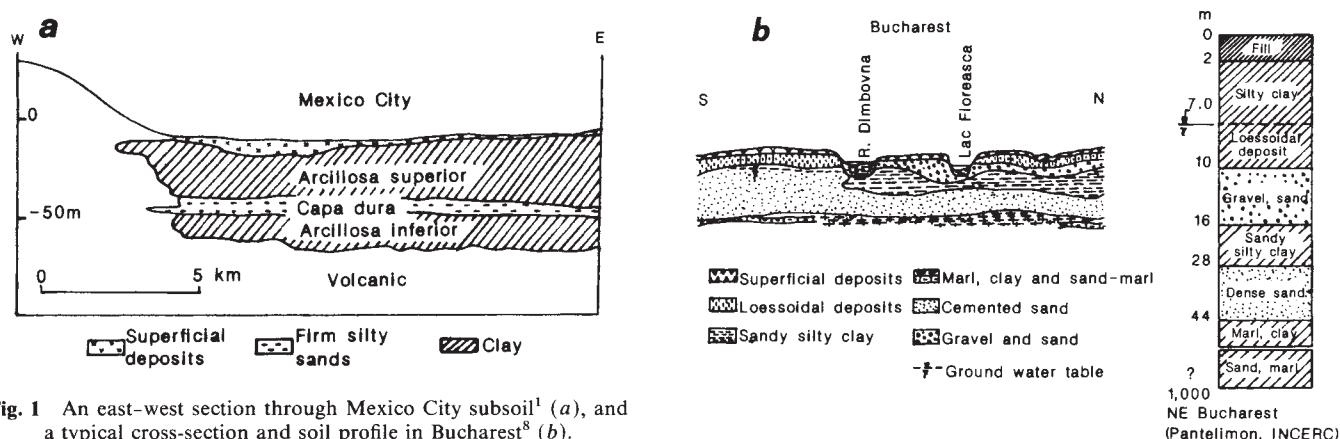


Fig. 1 An east-west section through Mexico City subsoil¹ (a), and a typical cross-section and soil profile in Bucharest⁸ (b).

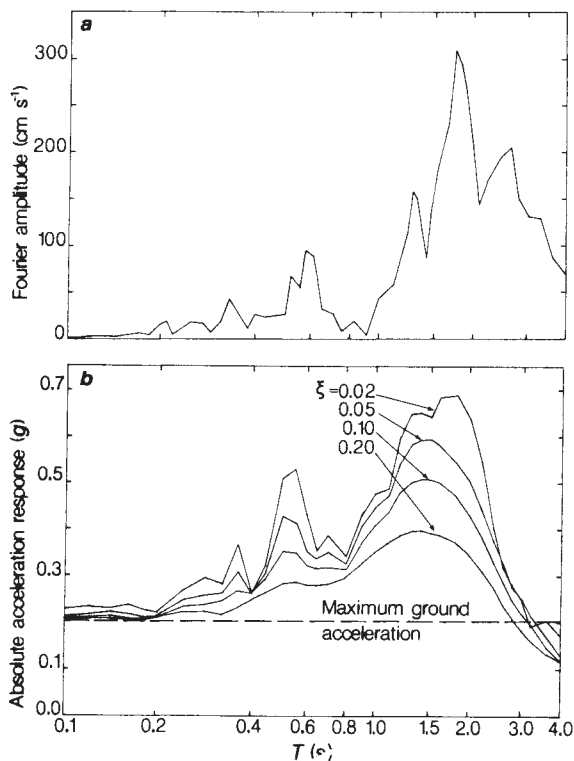


Fig. 2 *a*, Fourier amplitude spectrum and *b*, NS response spectra of the Romanian earthquake on 4 March 1977.

central Romania, for example, is a confined territory of $\sim 2,000 \text{ km}^2$ in which about 50 seismic events with magnitudes greater than 5 have occurred since the beginning of the last century. Four of these events, in 1802, 1929, 1940 and 1977, were especially destructive. For the 1977 event a substantial body of data is available^{8,9}, with both seismological and engineering significance. As in the recent Mexico City and Chilean earthquakes, the long-period components of the ground motion were amplified at long distances, especially through the alluvial deposits in southern Romania and Bulgaria. The Fourier amplitude spectrum of the north-south (NS) component, recorded at the Romanian Building Research Institute (INCERC) in Bucharest, is shown in Fig. 2*a*, and the corresponding soil profile in Fig. 1*b*. The peak recorded ground acceleration was 0.20g. Acceleration response spectra for the same earthquake are plotted in Fig. 2*b* for critical damping ratios $\xi = 0.02, 0.05, 0.10$ and 0.20; the response spectrum is an engineering representation of earthquake ground motion in terms of the variation of the peak response of a simple single-degree-of-freedom oscillator with natural period T (s), and damping ξ expressed as a proportion of critical damping. The greatest accelerations occur for periods > 0.8 s, and peak at ~ 1.5 – 2.0 s. The spectral acceleration is representative of the force experienced by a structure during the earthquake, and such long-period amplification is unusual when compared with standard spectra assumed for purposes of design in many countries^{12,13}.

The main geotechnical problem in

Bucharest arises from the presence of loessial (soft, wind-deposited) soils. In some areas, in the presence of water, these soils settle as much as 2 m under their own weight, thus any kind of foundation construction on such soils poses a difficult problem for geotechnical engineers. A soil amplification study⁸ based on analysis of nonlinear wave propagation through the soil profile in Fig. 1*b* demonstrated that the base-rock acceleration, with an amplitude estimated at 0.03g, is magnified about seven times, yielding a maximum surface acceleration of $\sim 0.20\text{g}$ as recorded in the NS component. The predominant period of vibration of the soil was computed at ~ 1.2 s.

By far the greatest structural damage in Bucharest was suffered by relatively light, reinforced concrete buildings 8–14 storeys high with natural periods between 0.9 and 1.6 s. This observation confirms the indications given by the Fourier and response spectra of the earthquake record (Fig. 2*a* and *b* respectively). The relatively high stiffness of the massive reinforced-concrete structures, generally 6–8 storeys high, which are typical of post-war construction in the region, resulted in relatively short fundamental periods of vibration and consequently these buildings survived with little or no damage⁹. Similar damage reports have been published for the 1985 Mexico City earthquake¹, in which 106 reinforced-concrete-frame buildings collapsed and a further 36 were severely damaged. Only one of these buildings exceeded 15 stories in height, and 69% were constructed after 1957, when earthquake-resistant design regulations were first introduced.

Dynamic torsional coupling

The principles of building construction to withstand earthquakes are well established^{14,15}. First, the conceptual design must ensure a ductile structure which can absorb energy: for example, foundations must be designed to avoid any differential movement. Differential stiffness between piles and columns which could cause torsion or rocking should be avoided; stiff upper stories resting on a relatively 'soft', open ground-floor can lead to excessive stress in the base columns. There must also be adequate provision for energy absorption, for example by the inclusion of structurally redundant partition walls designed to fail during earthquakes and be replaced afterwards. Here I concentrate on research into the problems of building configuration, in particular where the design is asymmetrical, as when stiff elements such as the shear walls surrounding lift shafts are located eccentrically on plan. A typical arrangement is shown in Fig. 3*a*. In the present study I evaluate the margin of safety in the earthquake-resistant design of such buildings, termed 'torsionally coupled' as defined below, when building code provisions are adopted. In particular, the torsional design recommendations of the Mexican earthquake code² are evaluated for the response of a particular class of buildings to ground motion exhibiting long-period amplification, as for example in the record of the Romanian 1977 earthquake (Fig. 2*a*).

For the purposes of this article a torsion-

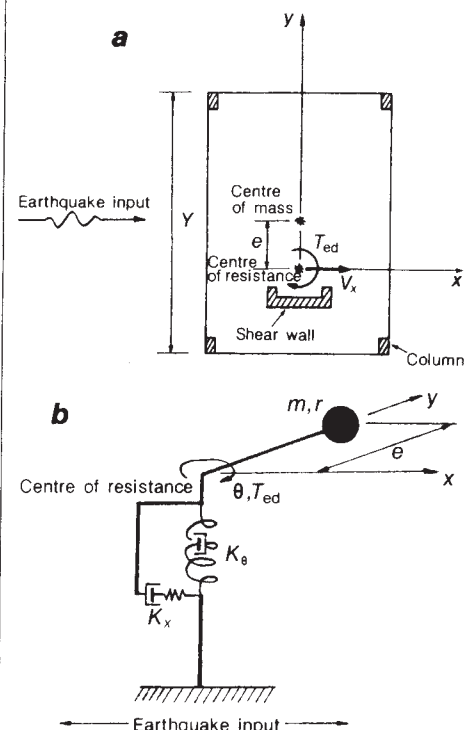
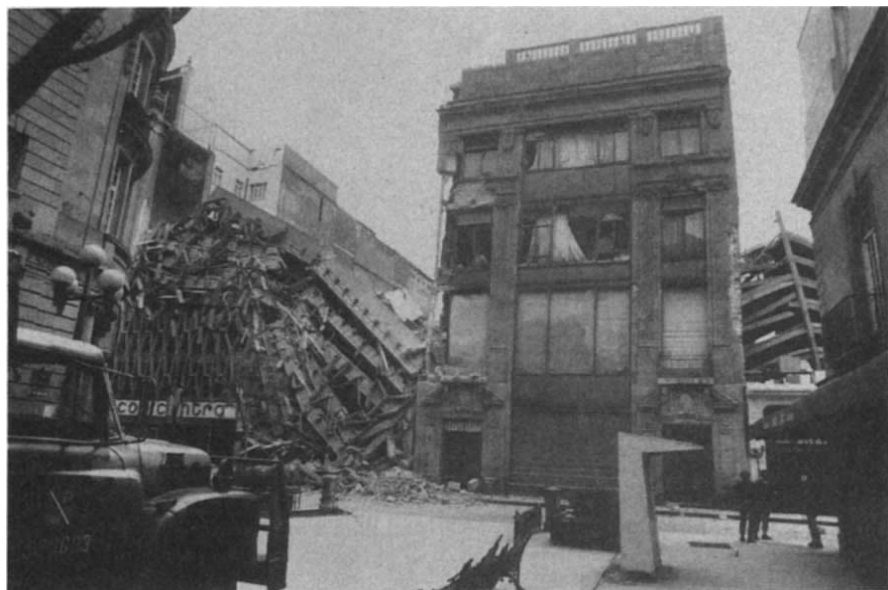


Fig. 3 *a*, Typical plan view of a torsionally coupled building with one eccentricity component; *b*, the corresponding model. K_x , Translational stiffness; K_θ , torsional stiffness.

ally coupled building is defined as one in which the centres of mass and resistance in at least one storey are not coincident. This means that the inertia forces (acting at the centre of mass) and the elastic forces (acting at the centre of resistance) form a dynamic couple which interconnects the translational and torsional response. In a torsionally uncoupled building, the centres of mass and resistance of all the stories lie on the same vertical axis and therefore translational and torsional responses are independent. The occurrence of torsional coupling is particularly evident in buildings¹⁶ in which the floor slabs act as diaphragms, infinitely rigid in their own plane: for example, solid, monolithic reinforced-concrete slabs supported on slender moment-resisting frames.

The distance between the centres of mass and resistance is referred to as the static eccentricity, or simply the eccentricity. This quantity may be determined by applying the principles of static engineering mechanics to a specified mass and stiffness distribution. Irregularities of this kind may arise, for example, from the functional architectural requirements of the building, an irregular plan configuration or an unbalanced distribution of the vertical structural elements. It has been recognized, however, in research work¹⁷⁻²⁰ and in building codes²¹ that there is an uncertainty about the magnitude of the eccentricity during an earthquake. Accidental eccentricities may result from a number of causes which cannot be quantitatively determined²²: variations between the actual and assumed mass and stiffness distributions; the presence and participation in the building response of stairs, partitions or masonry in-fill walls; nonlinearities, failures and consequent shifting in the structural response characteristics; displacement constraints from adjacent structures; the rotational component of ground motion about a vertical axis; and multi-support excitation.

Computer programs^{23,24} using complex multi-degree-of-freedom structural models have been developed to analyse the earthquake response of torsionally coupled buildings. Although the problem of torsional coupling in simple models was recognized and experimentally investigated in 1943 (ref. 25), it was not until 1958 that the first conclusion of practical significance was made²⁶, namely that the dynamic torque T_{ed} may be considerably larger than the product of the uncoupled shear force V_{x0} and eccentricity e . This conclusion has since been verified^{5,22,27-30} using more general models of torsionally coupled buildings. The present study is concerned with single-storey torsionally coupled buildings with centres of mass and resistance lying on the same principal axis of resistance (Fig. 3a, modelled mathematically as in Fig. 3b). Detailed parametric analyses of such buildings



An indication of the damage caused by the Mexico City earthquake. Above, precast concrete floor plates from a multi-storey car park have pushed sideways the adjacent old building. On the right, progressive collapse of the steel-framed Hotel de Carlo was contained after several stories were lost.



have already been made^{22,27-30}, so emphasis is placed here on a comparison with the torsional recommendations of building codes²¹, and the Mexican code² in particular.

The building is considered to consist of a rigid floor-diaphragm supported on structural framing and shear walls. The floor slab behaves as a rigid body in a general plane motion with two degrees of freedom: horizontal translation, and rotation about a vertical axis through the centre of resistance. Such buildings are commonly called 'shear' buildings because of the shape of their primary deformation mode. It is assumed that the mass of the building is lumped at the centre of mass, which coincides with the centroid of the floor slab. The structural elements supporting the slab and providing the lateral resistance are massless, elastic and viscously damped springs (see Fig. 3b). The investigation of single-storey buildings is justified because the response of multi-storey torsionally coupled buildings can, in many cases, be predicted^{31,32} from the response of associated single-storey buildings.

The earthquake ground motion is assumed to act in the x -direction only, and consists of the digitized NS Romanian 1977 ground acceleration record. The

coupled translational and torsional response components of the structure have been computed in the frequency domain using numerical integration methods^{22,33}, and are expressed parametrically in terms of the damping ratio ξ , the static eccentricity ratio $e_s = e/r$, where r is the radius of gyration of the floor mass m about the centre of mass, and the uncoupled frequency ratio $\lambda = \omega_\theta/\omega_x$ where ω_θ and ω_x are, respectively, the torsional and translational natural frequencies of the torsionally uncoupled building ($e=0$). Note that the translational natural period $T = 2\pi/\omega_x$ (Fig. 2b).

Comparison with Mexican code

In 1979, Rosenblueth³⁴ discussed a number of improvements which had been implemented in the Mexican code² as a result of research and of an interchange of ideas and experiences with the Applied Technology Council of the United States. Among the improvements was a more detailed treatment of design storey torques, intended to account for the dynamic amplification of eccentricity which results from torsional coupling, and based on earlier results by Rosenblueth and Elorduy²⁸. To this end, an amplification of 1.5 in the static eccentricity e was recommended to define the dynamic eccentricity e_d ; that is,

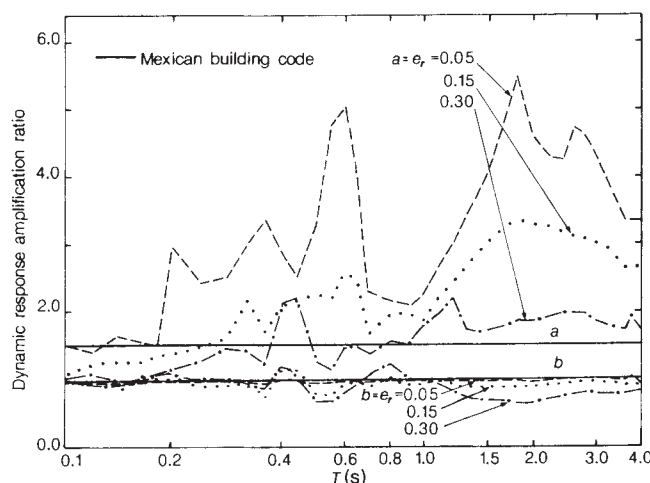


Fig. 4 Dynamic amplification of eccentricity (a) and shear (b) due to torsional coupling. $\lambda = 1.0$, $\xi = 0.05$.

the design storey torque T_{eb} was to be taken as $1.5eV_{x0}$, where V_{x0} is the design storey shear as recommended by all the various building codes²¹ and represents the storey shear, or lateral force, calculated for a building with the eccentricity neglected (that is, torsionally uncoupled). In addition, the design eccentricity was to be increased by $0.1Y$, where Y is the plan story dimension measured perpendicular to the direction of the applied earthquake forces (Fig. 3a). This additional term accounts for the probable accidental eccentricity e_a arising from the various sources listed earlier. Although this latter term was recognized to be a crude way of dealing with several variables, it was felt that such an approach was justified because, first, a more ambitious and therefore time-consuming and costly provision would meet with objections and probable rejection by practising engineers, and second, the current state of knowledge would not realistically justify a more refined treatment. In summary, the recommendations of the Mexican 1977 code² for the design torque T_b are

$$\begin{aligned} T_b &= T_{eb} + T_a \\ &= (e_d + e_a) V_{x0} \\ &= (1.5e + 0.1Y) V_{x0} \end{aligned}$$

The development of equivalent static methods for incorporating torsional effects in building design stems from the work of Bustamante and Rosenblueth⁵, who first introduced the concepts of dynamic eccentricity and the dynamic amplification of static eccentricity as described above. These concepts were later implemented in the majority of building codes. Although compilers of building codes were quick to adopt the dynamic amplification concept, it has become apparent from the work of various investigators^{22,27,28,30} that this method does not provide a complete (or conservative) description of the effects of torsional coupling over the full ranges of the controlling parameters which define the dynamic torsional characteristics of typical buildings. The studies have shown that when the

eccentricity is small ($e \ll Y$), the dynamic amplification of eccentricity can be well in excess of 1.5, but that when such large amplification occurs (that is, in particular when the uncoupled natural frequencies ω_θ and ω_x are close, so that $\lambda \approx 1.0$) the story shear V_x is smaller than the design value V_{x0} , hence the dynamic amplification of shear is < 1.0 .

The torsional provisions of the Mexican code, in common with most codes, take no account of the effects of the frequency ratio λ or of the level of damping ξ present in the structure. The results presented in Fig. 4 relate to the worst-case situation for design (that is, $\lambda = 1.0$) and further assume $\xi = 0.05$, a value which is conservative for the majority of buildings. A study by Hart *et al.*¹⁸ has shown that of 19 buildings investigated, 9 had fundamental torsional frequencies within 25% of the corresponding lateral frequencies; hence, torsional coupling probably has a significant effect on the earthquake response of many buildings, particularly since greatest amplification of eccentricity occurs when the static eccentricity ratio e_r is small.

Figure 4 illustrates the effects of torsional coupling on the dynamic amplification of shear and eccentricity for $e_r = 0.05, 0.15$ and 0.30 in the response to the NS Romanian 1977 earthquake, clearly demonstrating the implications of long-period amplification of ground motion on the magnitude of coupling effects. In particular, for fundamental (uncoupled) building periods $T > 1.0$ s and for $e_r = 0.05$, the dynamic eccentricity is amplified compared with the static value by a factor of up to 5.5, in comparison with the building code estimate of 1.5 as shown. Furthermore, the dynamic shear is approximately equal to the uncoupled (design) value V_{x0} in this range. Hence the dynamic forces on the building will in combination exceed design values to an extent which may result in the widespread damage or failure of buildings with fundamental periods in this range; that is, buildings that are 10–15 storeys high¹⁸. This observation coincides with the reported

damage to buildings in both the Chilean⁷ and Mexican earthquakes³⁵ (1985), in which torsional effects were cited as a major cause of failure, as in the Romanian earthquake itself^{8,9}.

The deficiency of the torsional recommendation of the Mexican code is less evident for larger eccentricities, when the dynamic amplification of eccentricity assumes smaller values (Fig. 4). Nevertheless, the provision underestimates to some extent the dynamic torque even for large static eccentricity ($e_r = 0.30$), especially when $T > 0.9$ s. However, in the same range the dynamic shear is reduced to ~60–70% of the design value V_{x0} and will thus offset to some extent the underestimation of dynamic torque.

Conclusions

Early reconnaissance reports following the 19 September 1985 earthquake in Mexico City gave strong indications that even those buildings that had been designed and built strictly in accordance with the local earthquake codes and modern engineering practice, collapsed as a result of the earthquake. This may be explained, in part at least, by the unusual amplifying effects of the geological formations beneath the city. In addition, however, the present study shows that the earthquake design procedures accounting for torsional coupling in the Mexican code underestimate the effect within particular ranges of the controlling parameters defining the dynamic characteristics of buildings. In particular, torsional coupling is more significant in the response of asymmetric buildings to an earthquake whose spectrum exhibits a concentration of energy in the range of natural periods typical of many modern multi-storey buildings of medium height and of conventional construction. Because of the uncertainties surrounding the precise distribution of mass and/or stiffness, even buildings which are nominally symmetric can respond with strongly coupled lateral and torsional modes of vibration¹⁹, resulting in an amplification of torsional response which significantly exceeds the design value in the Mexican building code.

The lessons learnt in the wake of the Mexico City disaster³⁶ must be followed by a detailed re-examination of earthquake-resistant design principles as indicated by the present research, followed by worldwide enforcement through more stringent design and supervision building codes in seismic zones. □

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1. Winney, M. *New civ. Engr.* 17–18 (3 October 1985).
2. National University of Mexico. *Design Manual for Earthquake, According to the Construction Regulations for the Federal District of Mexico* 406, Ch. 37, Art. 240(VII) (1977).
3. *Financial Times* (21 September 1985).

4. Rosenblueth, E. *Proc. 2nd World Conf. Earthquake Engng.* Japan, 1 (1960).
5. Bustamante, J. I. & Rosenblueth, E. *Proc. 2nd World Conf. Earthquake Engng.* Japan, 2, 879-894 (1960).
6. Scawthorn, C., Iemura, H. & Yamada, Y. *Earthquake Engng. struct. Dynam.* 9, 93-115 (1981).
7. Booth, E. *New civ. Engr.* 16-17 (4 April 1985).
8. Tezcan, S. S., Yerlici, V. & Durgunoglu, H. T. *Earthquake Engng. struct. Dynam.* 6(4), 397-421 (1978).
9. Berg, G. V., Bolt, B. A., Sozen, M. A. & Rojahn, C. *Earthquake in Romania 4 March 1977: An Engineering Rep.* (National Academy Press, Washington DC, 1980).
10. Tezcan, S. S. & Ipek, M. *Earthquake Engng. struct. Dynam.* 1, 203-215 (1973).
11. Berg, G. V. *The Skopje, Yugoslavia Earthquake 26 July 1963* (American Iron and Steel Institute, New York, 1964).
12. Newmark, N. M., Blume, J. A. & Kapur, K. K. *J. Pwr. Div. Am. Soc. Civ. Engrs* 99, No. P02, 287-303 (1973).
13. Berg, G. V. *Seismic Design Codes and Procedures* (Earthquake Engineering Research Institute, Berkeley, California, 1982).
14. Dowrick, D. J. *Earthquake Resistant Design* (Wiley, London, 1977).
15. Rosenblueth, E. *Design of Earthquake-resistant Structures* (Pentech, London, 1980).
16. *Seismic Design for Buildings*, TM-5-809-10, 23-26 (US Departments of Army, Navy and Air Force, Washington, DC, 1973).
17. Douglas, B. M. & Trubert, T. E. *Bull. seism. Soc. Am.* 63(3), 1025-1039 (1973).
18. Hart, G. C., Di Julio, R. M. & Lew, M. J. *struct. Div. Am. Soc. Civ. Engrs* 101, No. ST2, 397-416 (1975).
19. Jennings, P. C., Matthiesen, R. B. & Hoerner, J. B. *Earthquake Engng. struct. Dynam.* 1(2), 107-132 (1972).
20. Newmark, N. M. *Proc. 4th World Conf. Earthquake Engng.* Santiago, Chile, 2, Pt A3, 19-32 (1969).
21. *Earthquake Resistant Regulations, A World List* (International Association for Earthquake Engineering, Tokyo, 1984).
22. Chandler, A. M. thesis, Univ. London (1985).
23. Wilson, E. L. & Dovey, H. H. *Static and Earthquake Analysis of Three-dimensional Frame and Shear Wall Buildings—ETABS* University of California, Berkeley Rep. EERC 72-01 (1972, revised 1977).
24. Wilson, E. L. *et al. Extended Three-dimensional Analysis of Building Systems—ETABS* University of California, Berkeley Rep. EERC 75-13 (1975).
25. Ayre, R. S. *Bull. seism. Soc. Am.* 33(2), 91-119 (1943).
26. Housner, G. W. & Outinen, H. *Bull. seism. Soc. Am.* 48(2), 221-229 (1958).
27. Kan, C. L. & Chopra, A. K. *J. struct. Div. Am. Soc. civ. Engrs* 103, No. ST4, 805-819 (1977).
28. Rosenblueth, E. & Elorduy, J. *Proc. 4th World Conf. Earthquake Engng.* Santiago, Chile, 1, Pt A1, 185-196 (1969).
29. Dempsey, K. M. & Irvine, H. M. *Earthquake Engng. struct. Dynam.* 7(2), 161-180 (1979).
30. Tsienias, T. G. & Hutchinson, G. L. *Proc. Instn civ. Engrs* 71, 821-843 (1981).
31. Kan, C. L. & Chopra, A. K. *Earthquake Engng. struct. Dynam.* 5(4), 395-412 (1977).
32. Gluck, J., Reinhorn, A. & Rutenberg, A. *Proc. Instn civ. Engrs* 67, 411-424 (1979).
33. Hall, J. F. *Earthquake Engng. struct. Dynam.* 10, 797-811 (1982).
34. Rosenblueth, E. *Earthquake Engng. struct. Dynam.* 7(1), 49-61 (1979).
35. Winney, M. *et al. New civ. Eng.* 4-5 (26 September 1985).
36. Berg, G. V. & Hanson, R. D. *Proc. 5th World Conf. Earthquake Engng.* Rome, Session 1A (1973).

ARTICLES

DNA bending at adenine·thymine tracts

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Intrinsic bending of DNA molecules results from local structural polymorphism in regions of homopolymeric dA·dT which are at least 4 base pairs long; the A·T tracts must be repeated in phase with the helix screw. Bending, in the direction of base-pair tilt rather than roll, occurs at the junctions between the A·T tract and adjacent B-DNA, with a larger angle at the 3' than at the 5' end of the A tract.

SEQUENCE-directed bending of the DNA helix is now generally thought to be the origin of the anomalously slow gel electrophoretic mobility of DNA fragments isolated from the kinetoplast body of tropical parasites¹⁻⁷, and from other sources such as the origin of replication of bacteriophage λ (ref. 8). Wu and Crothers⁶ used the variation in gel mobility to map a bending locus contained in a *Sau3A* restriction fragment isolated from *Leishmania tarentolae* kinetoplast DNA, and identified the sequence

-GAATTCCCAAAATGTCAAAAATAGGCAAAAATGCCAAAATCCCAAC-
↑
bend centre

at the bending locus. The striking feature observed is a regular repeat of the sequence element $CA_{5-6}T$ with 10-base pair (bp) periodicity around the centre of the bend. Presumably each A_n tract produces a small bend in the DNA helix axis; repetition of these elements in phase with the helix screw results in their coherent addition to form a large overall bend. The objective of the work reported here is to explore this model, including the predicted role of phasing, the possible importance of increased helix flexibility, the strictness of the requirement for a continuous run of A residues, and the potential role of the junctions with other bases flanking the A tracts.

We approached the problem by chemical synthesis of appropriate oligonucleotides (Table 1) and their complementary strands, adjusted so that the resulting duplexes contained single-stranded regions at the ends which allowed ligation in a unique polarity to form longer DNA molecules containing up to 30 repeats of the oligonucleotide. Gel mobility anomalies in the multimers were assayed by running the ligation products on polyacrylamide gels, with ligated 10-bp *Bam*HI linkers as size standards. It is expected that increased bending, with the accompanying decrease in DNA end-to-end distance, should lead to

decreased gel-electrophoretic mobility^{9,10}. We define the apparent length of each multimer as being equal to the length of the marker DNA having the same mobility; the experimentally determined ratio R_L of apparent length to real length is assumed to be an increasing function of the extent of bending.

Influence of phasing

The role of phasing of the bending loci was addressed by examining a set of sequences $(A_5N_k)_n$ ($k = 4, 5, 6, 7, 10$), each of which contains the tract A_5 , flanked 3' and 5' by C, with a total of k bases intervening in the G+C-rich segment between the A_5 tracts. As shown in Figs 1 and 2, the gel mobility is strongly affected by the phasing of the A_5 tracts. Minimum mobility is seen for the series $(A_5N_5)_n$ in which the 10-bp phasing nearly matches the expected helix screw of about 10.3 bp per turn, calculated from the average of 10.5 for B-DNA and 10.1 for poly(dA)·poly(dT) in solution¹¹⁻¹³. A substantial but smaller anomaly in electrophoretic mobility is seen for the series $(A_5N_6)_n$, while $(A_5N_4)_n$ and $(A_5N_7)_n$ behave more normally. Hence, the results confirm the prediction that bending elements must be repeated in phase with the helix screw in order to add coherently.

The normal gel mobility of the series $(A_5N_{10})_n$ is important for distinguishing between bending due to increased flexibility and systematic bending in which the direction of the helix axis is altered in a definite way. Suppose, for example, that A_n tracts have increased flexibility because of relatively easier bending by roll into the major and minor grooves. Such bending is predominantly in a fixed plane, so the effects should sum coherently if the A_n units are separated by either an integral or a half-integral number of turns. Since the experiments show no bending when the separation is half-integral, we conclude that the bends have directional preference, and the series $(A_5N_{10})_n$ has normal mobility because it forms a zig-zag structure in which

the systematic bends are nearly exactly out of phase. Recent hydrodynamic results also show that the bending locus is not characterized by increased flexibility¹⁴

Interruptions in the A tract

The importance of a continuous run of A residues for the bending phenomenon was investigated by interrupting the A₅ tract with another nucleotide N (sequence code IAN) at the central base. Figure 3 shows that these single base-pair changes cause the gel mobility to revert nearly to normal, from which we conclude that bending is almost abolished. Hence, a continuous run of A residues is the basis for the bending phenomenon; clearly, the purines A and G are not equivalent in this case.

The base least effective in interrupting the influence of a run of As is T; this observation may be of interest in considering possible DNA structural polymorphism in the TATA box region of promoters. In addition, we found that the sequence (G₅N₅)_n has normal gel-electrophoretic mobility (data not shown),

confirming the special role of A tracts (or T tracts if the complementary strand is examined).

Alterations in length of A tracts

To determine the influence of the length of the A tract, we synthesized the series (A_jN_{10-j})_n, in which the sequence phasing is kept constant at 10 bp, while the number of A residues in the continuous block is varied. The results collected in Fig. 3 show that an A tract of length 3 produces only slight electrophoretic anomalies; the effect is substantial with 4 As in a row, and increases to a maximum for a tract of length 6. Apparent bending is lessened but still substantial for (A₈N₂)_n and (A₉N₁)_n. In

Table 1 Sequences examined

Name	Sequence (5' → 3')
A ₅ N ₄	C A A A A C G G
A ₅ N ₅	G G C A A A A C G
A ₅ N ₆	G G C C A A A A C G
A ₅ N ₇	G G C C A A A A C C G
A ₅ N ₁₀	C C G G C A A A A C G G G C
IAC	G G C A A C A A C G
IAG	G G C A A G A A C G
IAT	G G C A A T A A C G
FCT	G G C A A A A A T G
FGG	C C G A A A A A G G
A ₃ N ₇	G G C C A A A C C G
A ₄ N ₆	G G C C A A A A C G
A ₆ N ₄	G G C A A A A A C
A ₈ N ₂	C C A A A A A A A
A ₉ N ₁	C A A A A A A A A
G ₅ N ₅	T C G T G G G G C
A ₅ -8	C C A A A A A C G G G C A A A A A A A
A ₈ -5	C C A A A A A A A C G G G C A A A A A
A ₆ -T ₆	A A A A C G G G T T T T T G G G C A A
A ₂₀	A A A A A A A A A A A A A A A A A A

Only one strand of each duplex is shown. All duplexes were constructed with 2-bp protruding 5' ends, except A₅N₄, which had 1-bp protruding 5' ends. Multimers of dA₂₀·dT₂₀ were prepared both by ligation of the duplex and, for sequences 100, 140 and 160 bp long, by ligation of dA₂₀ (or dT₂₀) on poly(dT) (or poly(dA)) followed by gel purification of the selected radiolabelled multimers and hybridization with the complementary strand of equal length. This procedure was followed because we could not be certain of the polarity of ligation of dA₂₀·dT₂₀. A₅N₄, A₅N₅ and FGG were synthesized manually using the phosphotriester method and the remainder of the sequences were produced by automated DNA synthesis using phosphoramidite reagents on an Applied Biosystems instrument. A sequence A₇N₃ was synthesized, but since two multimeric ladders of differing gel mobilities were produced, depending on the conditions of ligation, those results are not reported here. Ligated multimers of A₅N₄ and A₅N₅ were cloned, and their sequence was verified by Maxam-Gilbert sequencing reactions.

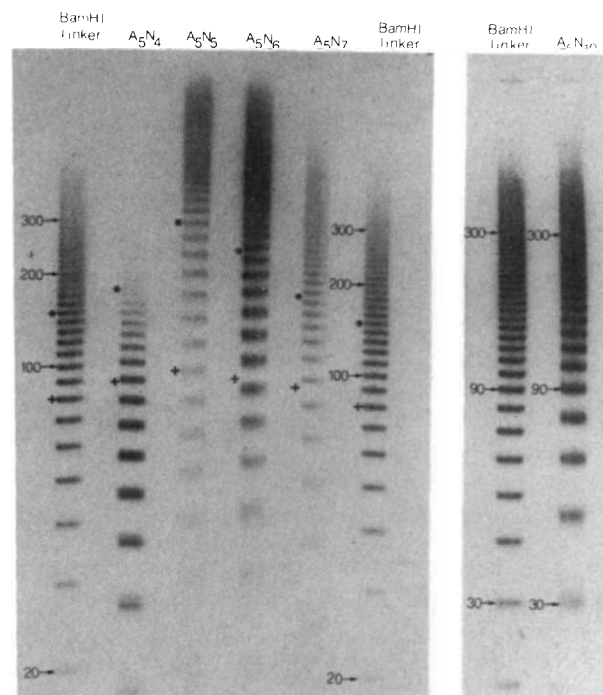


Fig. 1 Autoradiogram of the multimer series (A₅N_k)_n ($k = 4, 5, 6, 7, 10$) electrophoresed on a non-denaturing 8% polyacrylamide gel. Mobility indicators, interpolated where necessary, are: +, multimers of 80-bp sequence length; *, 150-bp sequence length. Chemically synthesized oligonucleotides (Table 1) were purified on a 20% polyacrylamide gel, followed by high salt elution from a DE-52 ion-exchange column. Purified oligonucleotides were desalted using Sep-Pak C18 cartridges (Waters). 8 μ g of each oligomer was 5'-labelled with 10 μ Ci of [γ -³²P]ATP (5 μ Ci pmol⁻¹; Amersham) in 10 μ l of solution, at 37 °C for 15 min. The reaction solution contained 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM dithiothreitol (DTT) and 6 U polynucleotide kinase (New England Biolabs). After labelling with [γ -³²P]ATP, 50 nmol cold ATP and an additional 4 U of kinase were added, and the reaction volume was doubled to 20 μ l. Kinasing with cold ATP was continued in the same buffer and temperature as above, for a period of 1 h. Two kinased oligonucleotides of complementary sequence (except for single-stranded residues at the 5' ends) were mixed, heated to 55 °C and cooled slowly to 0 °C to form hybrids. To 10 μ l of the hybridized mixture was added 800 U of T4 DNA ligase (New England Biolabs), and the reaction volume was adjusted to 20 μ l; the ligation mixture had the same concentration of Tris-HCl, MgCl₂ and DTT as the kinasing step, plus 2.3 mM cold ATP. The ligation reaction was allowed to proceed on ice overnight, after which it was quenched by addition of EDTA (pH 8.0) to 25 mM final concentration. Ligated products were run on non-denaturing 8% polyacrylamide gels (mono:bis acrylamide ratio = 29:1; 90 mM Tris-borate, 2.5 mM EDTA, pH 8.3) until the bromophenol blue dye had migrated 22 cm. The applied voltage was 7 V cm⁻¹, and electrophoresis was carried out at room temperature. Multimers of the BamHI linkers (10 bp, New England Biolabs) were also electrophoresed adjacent to a pBR322-HinfI digest (data not shown) to confirm their normal gel mobility.

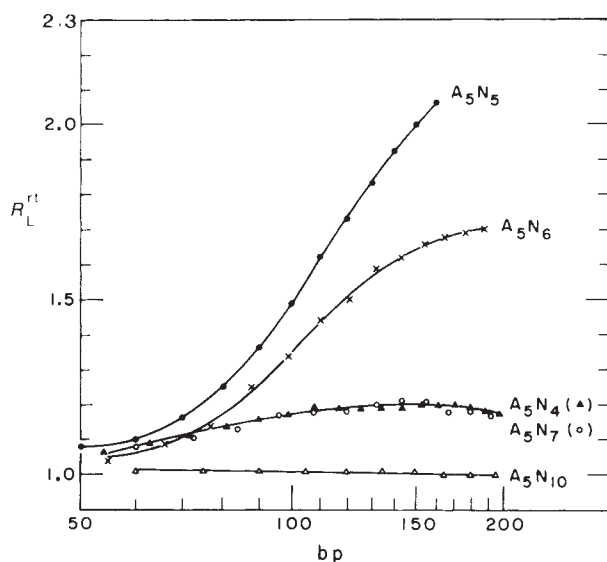


Fig. 2 The ratio R_L^r (at room temperature) of apparent multimer size, determined from comparison with the electrophoresis markers, to actual chain length. Increasing R_L^r indicates an anomalous structure which is interpreted as DNA bending. Strong anomalies are seen only when the sequence phasing of the A₅ tract is 10 (A₅N₅) or 11 (A₅N₆), corresponding to the number of base pairs in one double helical turn of DNA. Phasing of the A tracts at 1.5 helical turns (A₅N₁₀) produces no detectable bending, eliminating flexibility as an explanation for the phenomenon. Gels were run at room temperature.

order to be certain that the anomalous properties of these latter two sequences were not due to their high content of homopolymeric poly(dA)·poly(dT), we also examined the series (A₂₀)_n and found (Fig. 3) that these homopolymeric double helices have slightly increased electrophoretic mobility.

An interesting feature of the results in Fig. 3 is the crossover between the mobility curves for (A₅N₅)_n and (A₈N₂)_n at about 130 bp, with the latter showing greater mobility anomalies for molecules longer than the crossover size. We ascribe this behaviour to the strong influence of exact phasing on mobility anomalies: since (A₈N₂)_n has the higher content of homopolymeric poly(dA)·poly(dT), its helix screw is more closely matched to the 10-bp sequence repeat characteristic of this series of molecules. A similar explanation can be applied to the crossover

observed between the two series of polymeric A₄N₆ and A₉N₁ molecules.

Differential phasing of junctions

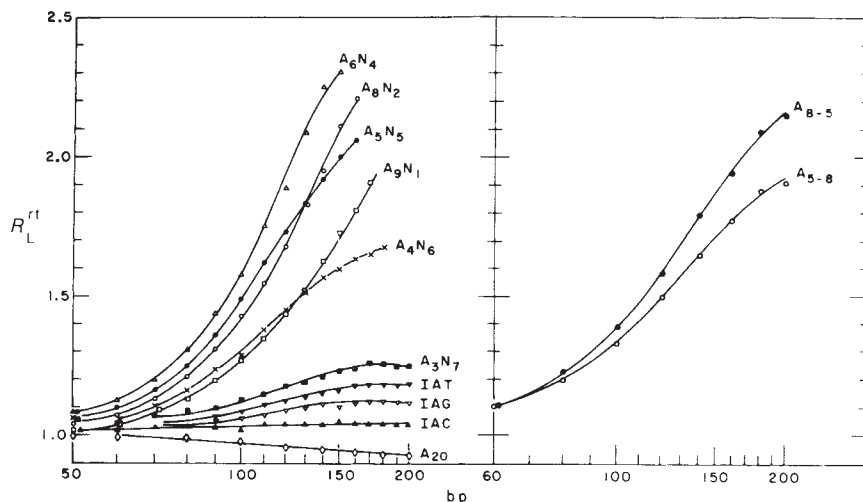
The substantial electrophoretic anomaly of (A₉N₁)_n is surprising because the 3' and 5' ends of the A tract are nearly a full helical turn apart; if the junctions produced equivalent bends in opposite directions, as, for example, proposed for the junction of an A-DNA segment with B-DNA¹⁵, one would expect the bends to compensate for each other when placed one helical turn apart. This observation raises the possibility that the bend is concentrated primarily at a single site, for which the only logical choices are the 3' and 5' junctions of the block of As. Therefore, we synthesized the series A₅₋₈ and A₈₋₅, which differ in that the former maintains a uniform 10-bp phasing of the 5' ends of the A tracts, with an alternating 7- and 13-bp separation between the 3' ends, whereas the latter has uniform 10-bp phasing of the 3' ends, with the 5' ends now characterized by the 7- and 13-bp alternating separation. Figure 3 shows that A₈₋₅ is clearly more anomalous, and hence more strongly bent, than A₅₋₈. We conclude that accurate phasing of the 3' ends of the A tracts is a dominant factor in bending; it follows that a substantial portion of the bending is concentrated at the 3' junction. However, the 5' junction must also contribute, as bending is maximized when both 3' and 5' junctions have 10-bp phasing, and are approximately half a helical turn (6 bp) apart, as in (A₆N₄)_n.

We investigated the symmetry properties of the bending locus through synthesis of (A₆T₆)_n in which every other CA₆C element is inverted to GT₆G. These molecules, with $R_L[140 \text{ bp}] = 2.13$, are nearly as anomalous as (A₆N₄)_n. We conclude that 2-fold rotation of an A₆ bending locus does not substantially alter the direction of bending.

Role of flanking bases

Given the importance of the junctions of the A tracts, it might be expected that the extent of bending would depend on the flanking bases. We found this to be the case, although the effects are not dramatic. Among the limited set of sequences examined, the greatest degree of bending ($R_L[150 \text{ bp}] = 2.07$ for sequence FCT; see Table 1) is seen when the 5'-flanking base is C and the 3' base is T, as found at the natural bending locus in *L. tarentolae*. The sequence with a flanking C both 3' and 5' is less bent ($R_L[150 \text{ bp}] = 2.00$ for A₅N₅), and the phenomenon is reduced further ($R_L[150 \text{ bp}] = 1.80$ for FGG) when G is present at both 3' and 5' ends.

Fig. 3 *a*, R_L^r (at room temperature) of multimers containing A tracts of different lengths, with constant sequence phasing. Values for the (A₂₀)_n series were obtained from the products of a ligation reaction carried out on (dA)₂₀·(dT)₂₀ as described in Fig. 1 legend. In addition, the mobilities were checked against (dA)_n·(dT)_n ($n = 100, 140, 160$) because of uncertainty about the polarity of ligation of (dA)₂₀·(dT)₂₀. We prepared (dA)_n by kinasing A₂₀, hybridizing with an equal concentration (by absorbance at 260 nm) of poly(dT) (Sigma) as template, and ligating as described for Fig. 1 at 4 °C overnight. Ligated multimers of A₂₀ were separated on an 8% denaturing polyacrylamide gel, and bands corresponding to the desired chain lengths were cut out, electroeluted, phenol-extracted and ethanol-precipitated. The corresponding (dT)_n oligomers were prepared analogously, using poly(dA) as template, and hybridized with the (dA)_n strand of equal length for gel analysis. *b*, R_L^r (measured at room temperature) for the sequences A₅₋₈ and A₈₋₅, which differ in phasing of the 5' and 3' junctions. The difference in gel anomalies for the two sequences demonstrates non-equivalence of the 3' and 5' junctions; the greater anomaly in A₈₋₅, in which the 3' junctions are phased at 10-bp intervals, implies greater bending at the 3' than at the 5' junction.



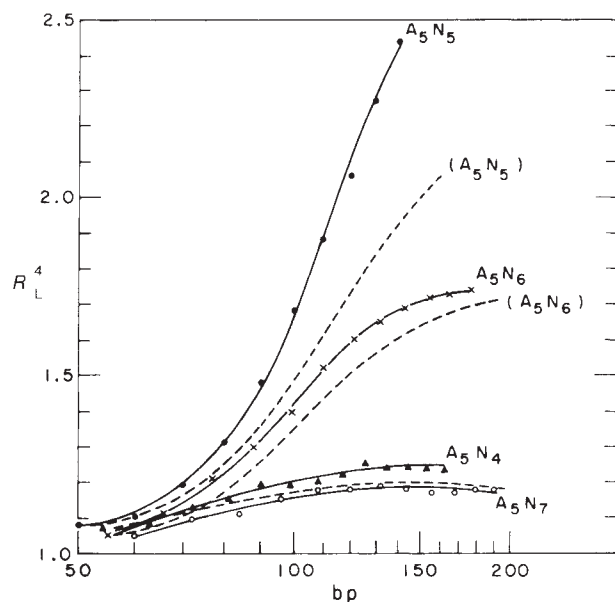


Fig. 4 Effect of lowered temperature on the gel mobility of the series $(A_5N_k)_n$, $n=4,5,6,7$. R_L^4 values are shown for an 8% polyacrylamide gel, run at 4°C, with voltage gradient 8 V cm^{-1} . The dashed lines show the results for gels run at room temperature (Fig. 2).

Temperature

It has been reported^{4,16} that increased temperature causes the gel mobility of kinetoplast DNA fragments to revert to more normal values, a property we found also to be characteristic of synthetic bending sequences. Figure 4 compares apparent lengths measured from gels run at 4°C and at room temperature for the series A_5N_k . Both of the highly anomalous series of molecules A_5N_5 and A_5N_6 become even more so at lower temperature, from which we conclude that bending is increased at lower temperature. However, the response of molecules longer than 100 bp is dramatically different in the two cases, implying a second effect in addition to an increase in the average bending angle; a possible explanation is the effect of lowered temperature on the DNA helical screw¹⁷, as discussed below.

Figure 5a, b summarizes the influence of lowered temperature on mobility anomalies of the series $(A_jN_{10-j})_n$. The molecules $(A_4N_6)_n$ and $(A_5N_5)_n$ are most strongly affected by lowered

temperature, to the extent that $(A_5N_5)_n$ becomes nearly as anomalous as $(A_6N_4)_n$, and retains greater anomaly than $(A_8N_2)_n$ even for molecules longer than 130 bp. Improved phasing match in $(A_5N_5)_n$ at 4°C doubtless contributes to this trend. The very weak bending observed for $(A_3N_7)_n$ at room temperature increases at 4°C, but remains much below that observed for other members of the series. Only $(A_9N_1)_n$ shows a general decline in the extent of mobility anomaly, for molecules between 60 and 150 bp in length, as temperature is decreased.

Models for DNA bending

We have identified two general classes of models for the origin of sequence-directed DNA bending: (1) models which assume that A tracts retain a normal B-DNA structure, and that bending can be explained in terms of nearest (or sometimes next-nearest) neighbour nucleotide sequences, and (2) models which invoke a longer-range structural polymorphism at the A tract, and which focus on the properties of junctions between it and adjacent B-DNA.

Class 1 includes the original analysis by Trifonov and colleagues^{18,19} of sequence periodicities which were proposed to produce 'curving' of eukaryotic DNA and thus ease its packaging in chromatin. Their approach identified the dinucleotides ApA (and TpT) which are clearly related to the A-tract bending phenomenon. The Dickerson-Calladine²⁰⁻²² analysis of base-pair roll resulting from purine-purine clash also falls into this category, although because of compensating roll at sequences adjacent to a purine-pyrimidine (Pu-Py) dinucleotide, the Dickerson²² parameters do not predict the bending observed in the kinetoplast DNA sequence³. On the other hand, the conformational analysis reported by Zhurkin and colleagues^{23,24} predicts that Pu-Py sequences should favour bending by compressing the minor groove, whereas Py-Pu sequences should bend into the major groove. Clearly, this model would predict systematic bending in the kinetoplast sequence with its 5-6-bp alternation of Pu-Py and Py-Pu nearest neighbours.

Models of class 2 take as their starting point the observation that poly(dA)·poly(dT) has an anomalous structure, as revealed by fibre diffraction²⁵, its 10.1-bp helical screw¹¹⁻¹³, its Raman spectrum^{26,27}, and its inability to be reconstituted into nucleosomes^{28,29}. Hence, it is possible that oligo(dA·dT) tracts in DNA can adopt one or more alternative structures in addition to the normal B configuration. One expects that the tendency to form the alternative structure will increase with the length of the A tract, because short runs of A do not gain enough free energy from the structural switch to compensate for the unfavourable free energy at the junctions with normal DNA. If the base pairs

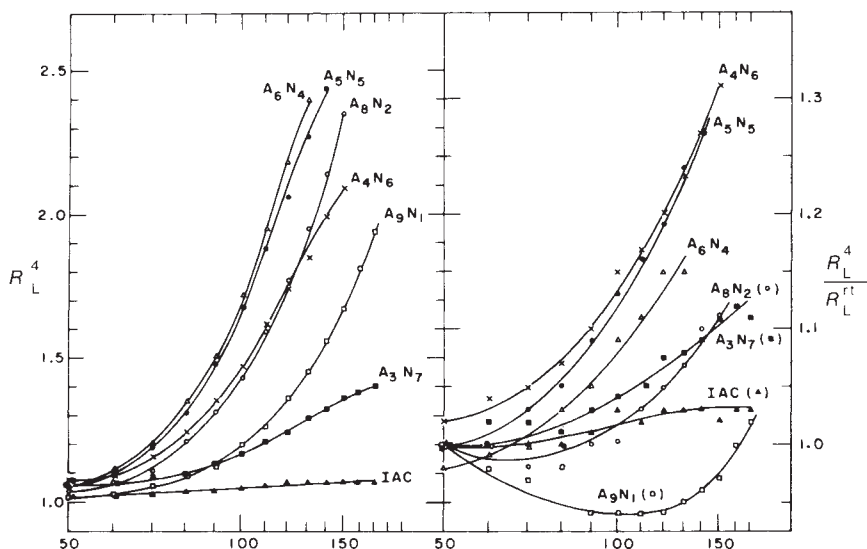


Fig. 5 Effect of lowered temperature on the mobility of the series $(A_jN_{10-j})_n$ and IAC (see Table 1), showing R_L^4 measured on a gel run at 4°C as in Fig. 4. b, Relative changes in R_L^4 values with temperature, shown as the ratio $R_L^4 / R_L^4(°)$.

are tilted in the alternative structure, as proposed from fibre diffraction for the 'heteronomous'²⁵ (H) structure of poly(dA)·poly(dT), then a bend in the helix axis is expected at the junction with B-DNA, as illustrated by models built to represent the junction of A- and B-DNA¹⁵. Note that the two strands in the H-DNA model are not equivalent, so there is no necessity for the 3' and 5' junctions of H-DNA with B-DNA to exhibit the kind of equivalence required for A-B junctions by the 2-fold structural symmetry of A- and B-DNA.

Interpretation of the results

The general requirement, characteristic of all the models, for phasing the A tracts in coherence with the helical repeat is clearly verified by our experiments. When that finding is combined with the lack of bending found for molecules in which the phasing is 1.5 helical turns, we can definitely exclude increased flexibility from the list of possible explanations for the observed electrophoretic anomalies.

Our results clearly favour models of class 2, in which runs of A of sufficient length adopt an alternative helical structure. This readily explains the requirement of at least four successive A residues in order to observe strong bending anomalies. In addition, loss of the bending phenomenon when A₅ is replaced by AAGAA eliminates all models that are based on purine clash or preferential bending at Pu-Py and Py-Pu sequences. In the model which invokes bending at ApA dinucleotides, ApA bends separated by half a helical turn cancel each other. On that basis one would expect that the bending in (A₃N₇)_n or in the IAN set, each of which has two putative ApA bending steps, should nearly equal the bending in (A₆N₁)_n, in which all but two of the ApA steps cancel. This prediction is definitely in disagreement with the facts. The essential problem with models of class 1 is that the bending observed in short A tracts is substantially less than expected. (We do not argue that small bends based on nearest-neighbour sequence do not occur, only that they cannot explain the A-tract bending phenomenon.)

The striking temperature dependence of bending is also readily explained in terms of the structural polymorphism model, by postulating that the equilibrium between B and the alternative structural form, provisionally called H, is shifted towards H at low temperature. This shift increases the average A-tract bending angle, and also affects the accuracy of helical phasing of the bending loci. Since junctions and therefore bends are absent when the A tract is in the B-form, higher temperature eliminates bending as the H-form disappears. Lowered temperature is expected to improve the phase match in (A₅N₅)_n and (A₄N₆)_n because conversion to the H-form brings the average helical screw closer to the 10-bp sequence repeat. In the case of (A₅N₆)_n, this factor works in the opposite direction because the helix is already overwound relative to the 11-bp sequence repeat, leaving only a modest increase in observed bending at low temperature, due to the increased average A-tract bending angle.

The unusual temperature dependence observed for A₆N₁ may result from a tendency of the H segment to include the single C residue, thus bridging continuously from one A tract to another. This would result in reduced average bending because H-B junctions would disappear, and would alter the otherwise expected increase of bending on lowering the temperature.

The observed non-equivalence in the importance of the 3' and 5' A-tract junctions for bending implies lack of 2-fold symmetry in the structure of the A tract. This supports the proposal that the structure may resemble H-DNA, whose most unusual feature is a lack of equivalence between the strands. A schematic model is shown in Fig. 6, based on the conformation of the dA strand in the H-form structure²⁵ and on the observation reported by Edmondson and Johnson³⁰ that T has a greater angle relative to the helix axis than does A in poly(dA)·poly(dT). It is a natural consequence of the model shown that bending is greater at the 3' than at the 5' junction of the A tract.

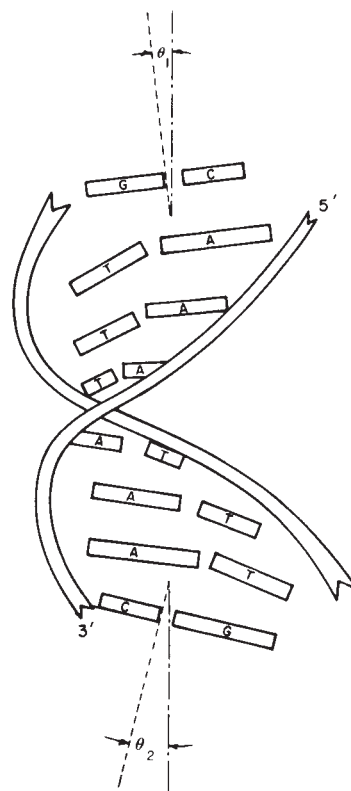


Fig. 6 Proposed model for differential bending at the 3' and 5' junctions. The A-tract helix backbone trajectories and tilt of the A residues are taken from the H-DNA structural model²⁵, and the extra tilt of the T residues is based on the optical experiments of Edmondson and Johnson³⁰. (—, Helix axis of the A-tract segment, assumed to be in the H-form structure; ---, helix axes of the adjacent B-DNA segments. At the 5' junction, the G·C base pair from the top B-DNA segment stacks parallel to the A base, rather than the T base, because of the stronger stacking interactions of A. At the 3' junction, the downward-tilted T residue prevents the G·C pair that begins the lower B-DNA segment from stacking parallel to the A base. As a consequence, the helix axis bending angle θ_2 is greater than the corresponding angle θ_1 . The pseudo-2-fold axis for the CA₆C segment lies in the plane of the paper, and runs through the centre of the A₆ tract; rotation about that axis produces no change in predicted bending, as is nearly true for the sequence A₆-T₆. The model is intended to be schematic; the bending angles θ and the base tilt angles have only qualitative significance.

The model in Fig. 6 also explains the observation that 2-fold rotation of every other CA₆C bending locus has little effect on the extent of bending. The pseudo-dyad axis in the model lies in the plane of the paper, and runs between the third and fourth A in the tract. The total bending in the direction of base-pair tilt at the two junctions as shown is not affected by rotation about the approximate 2-fold axis. However, if bending were in the direction of base-pair roll into the major groove at one junction and into the minor groove at the other (in a direction perpendicular to the paper in the model), 2-fold rotation would invert the direction of bending, and the effects of successive A and T tracts would cancel. Hence, models which rely on base-pair roll at H-B junctions can be eliminated.

Our results lead us to stress the potential general importance of local polymorphism of DNA structure. In the case of A-tract bending, a cooperatively induced, altered helical structure is clearly implied, with special effects at the 3' junction of the A tract with adjacent B-DNA. The recent observation by Reynolds *et al.*³¹ of specific antibiotic-induced cleavage at the 3' end of A tracts supports the view that DNA structure is significantly altered at that site. In addition, their observation that the same

drug cuts in the promoter TATA box suggests that a similar alteration may be possible there. Such structural variations may have wide significance for protein-DNA recognition.

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- Englund, P. T., Hajduk, S. L. & Marini, J. C. A. *Rev. Biochem.* **51**, 695-726 (1982).
- Marini, J. C., Levene, S. D., Crothers, D. M. & Englund, P. T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7664-7668 (1982).
- Levene, S. D. & Crothers, D. M. *J. biomolec. Struct. Dyn.* **1**, 429-435 (1983).
- Marini, J. C. *et al. J. biol. Chem.* **259**, 8974-8979 (1984).
- Ntambi, J. M. *et al. Molec. Biochem. Parasit.* **12**, 273-286 (1984).
- Wu, H. M. & Crothers, D. M. *Nature* **308**, 509-513 (1984).
- Hagerman, P. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4632-4636 (1984).
- Zahn, K. & Blattner, F. R. *Nature* **317**, 451-453 (1985).
- Lerman, L. S. & Frisch, H. L. *Biopolymers* **21**, 995-997 (1982).
- Lumpkin, O. J. & Zimm, B. H. *Biopolymers* **21**, 2315-2316 (1982).
- Peck, L. C. & Wang, J. C. *Nature* **292**, 375-378 (1981).
- Rhodes, D. & Klug, A. *Nature* **292**, 378-380 (1981).
- Strauss, F., Gaillard, C. & Prunell, A. *Eur. J. Biochem.* **118**, 215-222 (1981).
- Levene, S. D., Wu, H. M. & Crothers, D. M. *Biochemistry* (in the press).
- Selsing, E., Wells, R. D., Alden, C. J. & Arnott, S. J. *biol. Chem.* **254**, 5417-5422 (1979).

- Wu, H. M. thesis, Yale Univ. (1982).
- Depew, R. E. & Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4275-4279 (1975).
- Trifonov, E. N. & Sussman, J. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3816-3820 (1980).
- Trifonov, E. N. *Nucleic Acids Res.* **8**, 4041-4053 (1980).
- Drew, H. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 2179-2183 (1981).
- Calladine, C. R. *J. molec. Biol.* **161**, 343-352 (1982).
- Dickerson, R. E. *J. molec. Biol.* **166**, 419-441 (1983).
- Zhurkin, V. B., Lysov, Y. P. & Ivanov, V. I. *Nucleic Acids Res.* **6**, 1081-1096 (1979).
- Zhurkin, V. B. *J. biomolec. Struct. Dyn.* **2**, 785-804 (1985).
- Arnott, S., Chandrasekaran, R., Hall, K. H. & Puigjaner, L. C. *Nucleic Acids Res.* **11**, 4141-4155 (1983).
- Thomas, G. A. & Peticolas, W. L. *J. Am. chem. Soc.* **105**, 993-996 (1983).
- Jolles, B., Laigle, A., Chinsky, L. & Turpin, P. Y. *Nucleic Acids Res.* **13**, 2075-2085 (1985).
- Simpson, R. T. & Kunzler, P. *Nucleic Acids Res.* **6**, 1387-1415 (1979).
- Kunkel, G. R. & Martinson, H. G. *Nucleic Acids Res.* **9**, 6869-6888 (1981).
- Edmondson, S. P. & Johnson, W. C. Jr *Biopolymers* **24**, 825-841 (1985).
- Reynolds, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H. & Hurley, L. H. *Biochemistry* **24**, 6228-6237 (1985).

LETTERS TO NATURE

Large-scale anisotropy in the Hubble flow

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Detection of anisotropy in the Hubble flow is one of the outstanding problems of observational cosmology. It provides information on the mass density of the Universe and the extent to which the Universe is smooth. This, in turn, permits constraints to be placed on theoretical models of galaxy formation. Our approach to this problem has been to obtain infrared photometry for an all-sky sample of ScI-II galaxies identified by Rubin *et al.*¹ at a mean redshift of 5,100 km s⁻¹. At this distance these galaxies provide information on the Hubble flow well outside the Local Supercluster. From observations of half the sample, well distributed across the sky, we find a systematic streaming velocity of these galaxies of $\sim 1,000 \pm 300$ km s⁻¹. In the context of current speculation regarding non-baryonic particle species which may dominate the mass of the Universe, such large velocities on these spatial scales appear to exclude cold dark matter models.

We have used infrared photometry to measure the distances to this all-sky sample of galaxies. Infrared magnitudes have the advantage of being relatively unaffected by extinction and provide a reliable indication of distance when combined with either the optical-infrared colour-magnitude relation² or the infrared Tully-Fisher relation³. We have measured 45 of the 96 ScI-II galaxies comprising the Rubin *et al.*¹ 'minimum bias subset' in the J, H and K wavelength bands with a 35 arc s aperture using the 1.5-m Infrared Flux Collector in Tenerife. These 45 galaxies are well distributed across the sky (see Fig. 1). In particular, their redshift distribution is representative of that for the entire sample.

Our approach is to search for a systematic dipole velocity by comparing the velocities inferred from photometry with the observed recessional velocities. The analysis is carried out to find an apparent Local Group motion relative to the galaxy sample. Using the measured H magnitudes and the redshifts tabulated by Rubin *et al.*¹, we have carried out three different solutions for the apparent Local Group motion. For the first solution the H magnitudes were corrected for Malmquist bias in the sample and for the effect of using a fixed aperture. Both

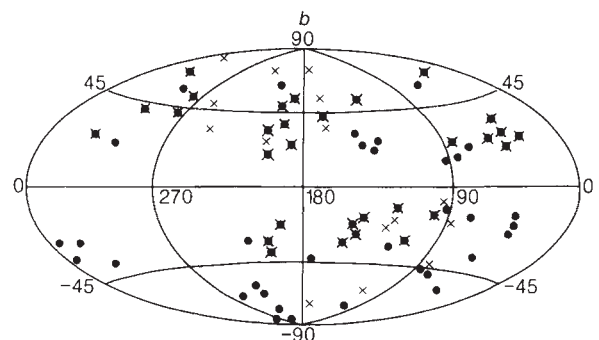


Fig. 1 Positions of galaxies observed in galactic coordinates on an equal-area projection of the celestial sphere. x, Galaxies observed in bands J, H and K; ●, galaxies detected by IRAS.

of these effects result in an apparent increase of galaxy luminosity with redshift. A 'composite' correction for these effects was determined from a plot of Hubble modulus (defined below) versus redshift. Hubble velocities were then derived from the corrected H magnitudes, assuming all the galaxies have the same absolute magnitude. Following the Rubin *et al.*⁴ approach, one need make no assumption about the precise value of the Hubble constant, H_0 , or the absolute magnitude, M , of the sample. Instead one uses the Hubble modulus, HM, defined as

$$HM = \log_{10} H_0 - 0.2 M - 5 = \log_{10} v_{\text{obs}} - 0.2 m$$

where v_{obs} and m are the redshift and apparent magnitude of a galaxy. The mean HM for the sample can then be used to find the Hubble velocity for each galaxy, assuming uniform expansion, from

$$v_H = 10^{(<HM> + 0.2 m)}$$

The difference between the observed redshift and the derived Hubble velocity, $v_{\text{obs}} - v_H$, and a putative systematic dipole velocity, v_D , are minimized in the least-squares sense, to derive a value for v_D . Because the redshifts have been corrected for the solar motion with respect to the Local Group (300 km s⁻¹ toward galactic longitude $l = 90^\circ$, latitude $b = 0^\circ$), v_D is the velocity of the Local Group relative to the frame defined by these galaxies. The result is shown in the first line of Table 1. Identical results, within the errors, are obtained using the J and K magnitudes.

In our second solution we used the H I 21-cm velocity widths and the infrared Tully-Fisher relation³ to find the relative lumi-

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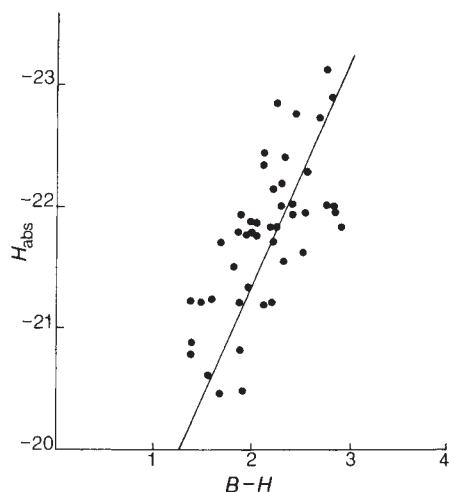


Fig. 2 Colour-magnitude relation for galaxies in the sample observed in bands J, H and K.

nosity of each galaxy. The solution using this relation is given in line 2 of Table 1. Evidently the resulting magnitude and direction of v_D are unchanged by using this third variable.

For a third solution we have used the $B-H$ versus H_{abs} colour-magnitude relation for this sample (Fig. 2) as a luminosity indicator. We find a slope of 1.85 ± 0.3 for this relation, consistent with slopes determined for other galaxy samples². The solution incorporating the colour-magnitude relation is given in line 3 of Table 1. Since the mean inclination of the sample is small ($\sim 35^\circ$), this solution should provide a better estimate of the Hubble velocities than the Tully-Fisher relation. The use of this third variable as an independent luminosity indicator has produced a solution essentially equivalent to the two previous ones. Thus, there is no bias in the absolute magnitudes of the galaxies in this sample sufficient to account for the velocity anisotropy observed. This is an important result which answers some of the criticisms of the original Rubin *et al.* study^{5,6}.

We have investigated the stability of this solution for a larger number of galaxies in the minimum-bias sample using 100- μm flux densities from the IRAS (Infrared Astronomy Satellite) catalogue⁷, which contains detections for 64 of the 96 galaxies comprising the sample. The distribution of these galaxies around the sky is also shown in Fig. 1. We have made corrections for Malmquist biasing similar to the 'composite' correction to the H magnitudes described above. Repeating the solution for the apparent Local Group motion using the IRAS 100- μm flux densities for these 64 galaxies gives the solution shown in line 4 of Table 1. We again find a solution which, within the errors, is identical to those found previously.

Line 5 of Table 1 contains the solution obtained by Rubin *et al.*⁴, using blue magnitudes without diameter correction (the correction for galaxy diameters was shown by Schechter⁸ to give a less reliable solution). This solution is identical, within the errors, to the infrared solutions. For comparison we have also listed the velocity inferred from the dipole anisotropy in the cosmic background radiation (CBR)^{9,10}.

The results in Table 1 demonstrate that the apparent Local Group motion relative to the reference frame defined by this group of galaxies is independent of the photometric data used, over a wavelength range of a factor of 200. Use of two independent luminosity indicators in the analysis does not change the solution. We conclude therefore that there is a genuine and substantial discrepancy between the apparent Local Group motion relative to this galaxy sample and that relative to the CBR.

Table 1 Solutions for the apparent Local Group motion

Solution	v_D (km s ⁻¹)	l (deg)	b (deg)
H magnitudes, 45 galaxies	621 ± 300	186 ± 30	-3 ± 29
H magnitudes with Tully-Fisher relation, 40 galaxies	846 ± 334	214 ± 40	-33 ± 34
H magnitudes with colour-magnitude relation, 45 galaxies	680 ± 330	184 ± 35	-36 ± 30
IRAS 100- μm , 64 galaxies	662 ± 220	190 ± 20	-6 ± 18
B magnitudes without diameter correction, 96 galaxies ⁴	586 ± 200	202 ± 19	-11 ± 17
Dipole anisotropy in the CBR ^{9,10}	600 ± 50	265	+35

The fact that the apparent Local Group motion found for this sample of galaxies is so different from that inferred from the CBR leads us to interpret our results in terms of a large-scale streaming motion induced by correspondingly large-scale density fluctuations in the mass distribution of the Universe. To find the magnitude and direction of the streaming motion for these galaxies, we must subtract the apparent Local Group motion, v_D , from the velocity of the Local Group as inferred from the CBR dipole anisotropy. Using the solution in line 3 of Table 1, we find a galaxy streaming velocity of 970 ± 300 km s⁻¹ toward $l = 305^\circ$, $b = 47^\circ$.

Is such a large-scale streaming consistent with other observations bearing on the large-scale structure of the Universe? Clutton-Brock and Peebles¹¹ have calculated the root-mean-square peculiar velocity of a spherical shell of galaxies in the redshift range of this galaxy sample, caused by density fluctuations consistent with the galaxy two-point correlation function. They find

$$\langle v^2 \rangle^{1/2} = 300 \Omega_0^{0.6} \text{ km s}^{-1}$$

where Ω_0 is the density parameter. This shows that, assuming that light traces mass, consistency between the two-point correlation function and our large streaming velocity requires a high-density Universe and precludes cosmologies with $\Omega_0 \ll 1$.

The streaming direction we find for this spherical shell of galaxies agrees with the streaming direction for the Local Supercluster (toward $l = 300^\circ$, $b = 25^\circ$) recently reported by Davies and Staveley-Smith¹², and is close to that found by Aaronson *et al.*¹³. This suggests a consistent picture in which the galaxies within a radius of 50 h^{-1} Mpc (for $H_0 = 100 h \text{ km s}^{-1} \text{ Mpc}^{-1}$) seem to be participating in the same overall streaming motion with respect to the fundamental cosmic reference frame.

Our result for this galaxy sample at a redshift of 5,000 km s⁻¹ does not confirm the extrapolation made by de Vaucouleurs and Peters¹⁴ that galaxies at redshifts $> 3,500 \text{ km s}^{-1}$ define a reference frame at rest with respect to the CBR. The Hubble flow is apparently not isotropic on scales as large as 50 h^{-1} Mpc.

Yahil *et al.*¹⁵ and Meiksin and Davis¹⁶ have analysed all-sky counts of IRAS galaxies to infer a large-scale mass dipole moment. The direction of this dipole moment is $35\text{--}50^\circ$ away from the direction of the streaming velocity we find. If the effective distance of the IRAS galaxies is well outside 50 h^{-1} Mpc, this rough agreement may be significant.

Large-scale galaxy streaming of this kind provides constraints on theoretical models for the formation of large-scale structure in the Universe. The scale sizes of the structures which first form in the gravitational instability model depend on the type of particle which dominates the mass of the Universe. This mass is currently thought to be in the form of some kind of 'hot' or 'cold' dark matter composed of exotic particle species¹⁷. The qualitative difference between these hot and cold dark matter

models, for present purposes, is the resulting mass fluctuation on large scales. In models dominated by cold dark matter it is difficult to produce streaming velocities as large as we have observed on scales of $50 h^{-1}$ Mpc. For example, Vittorio and Silk¹⁸ predict streaming velocities on this scale of $\leq 160 \text{ km s}^{-1}$ in models dominated by cold dark matter. On the other hand, if the dark matter is composed of hot particles, the mass fluctuation spectrum will have greater amplitude at large spatial scale, and will therefore tend to produce higher streaming velocities. Kaiser¹⁹ has calculated the galaxy streaming velocities in cosmologies dominated by one hot dark matter candidate, massive neutrinos. Substituting 700 km s^{-1} into his result for the predicted streaming velocity on the scale of the Rubin *et al.* galaxies gives a redshift of galaxy formation of $z \approx 5$. This is consistent with other observational constraints on the epoch of galaxy formation.

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1. Rubin, V. C., Ford, W. K. Jr, Thonnard, N., Roberts, M. S. & Graham, J. A. *Astr. J.* **81**, 687–718 (1976).
2. Tully, R. B., Mould, J. R. & Aaronson, M. *Astrophys. J.* **257**, 527–537 (1982).
3. Aaronson, M., Huchra, J. & Mould, J. *Astrophys. J.* **229**, 1–13 (1979).
4. Rubin, V. C., Thonnard, N., Ford, W. K. Jr & Roberts, M. S. *Astr. J.* **81**, 719–737 (1976).
5. Fall, S. M. & Jones, B. J. T. *Nature* **262**, 457–460 (1976).
6. Tammann, G., Yahil, A. & Sandage, A. *Astrophys. J.* **234**, 775–784 (1979).
7. *IRAS Point Source Catalog* (1984).
8. Schechter, P. L. *Astr. J.* **82**, 569–576 (1977).
9. Fixen, D. J., Cheng, E. S. & Wilkinson, D. T. *Phys. Rev. Lett.* **50**, 620–622 (1983).
10. Lubin, P. M., Epstein, G. C. & Smoot, G. F. *Phys. Rev. Lett.* **50**, 616–619 (1983).
11. Clutton-Brock, M. & Peebles, P. J. E. *Astr. J.* **86**, 1115–1119 (1981).
12. Davies, R. D. & Staveley-Smith, L. *Proc. ESO Workshop on the Virgo Cluster* (eds Richter, O.-G. & Binggeli, B.) 391–395 (European Southern Observatory, 1985).
13. Aaronson, M. *et al.* *Astrophys. J.* (in the press).
14. Vaucouleurs, G. de & Peters, W. L. *Astrophys. J.* **287**, 1–16 (1984).
15. Yahil, A., Walker, D. & Rowan-Robinson, M. *Astrophys. J.* **301**, L1–L5 (1986).
16. Meiksin, A. & Davis, M. *Astr. J.* **91**, 191–198 (1986).
17. Blumenthal, G. R., Faber, S. M., Primack, J. R. & Rees, M. J. *Nature* **311**, 517–525 (1984).
18. Vittorio, N. & Silk, J. *Astrophys. J.* **293**, L1–L5 (1985).
19. Kaiser, N. *Astrophys. J.* **273**, L17–L20 (1983).

Solar radio signatures suggestive of proton beams

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Electron beams propagating in the solar corona excite the ambient plasma to emit radio waves at the local plasma frequency and/or its harmonic. This general interpretation of type III radio bursts is well confirmed by *in situ* measurements. The rate at which peak flux drifts in frequency and time is a measure of the velocity of the exciter. Here we report a class of radio bursts with a significantly lower drift than normal; consequently, we show that they defy the usual interpretation. Possible exciters are slowly moving beams of protons.

A sensitive way of studying primary physical processes occurring on the Sun in relation to flares is to concentrate on simple events. It is impossible to study a single process in a large flare because of the multifarious mutual interactions of the various flare components. Consequently, we have studied a very small

and short-lived limb event observed on 10 July 1980 with the ETH Zurich digital radio spectrometer IKARUS¹. This instrument was operated with a frequency response of 3 MHz and a time resolution of 0.1 s. The flare was seen in soft (3.5–8.0 keV) and hard (28–490 keV) X-rays by the instruments on the Solar Maximum Mission as a spike of duration 61 s and 10 s (full width at half maximum), respectively. There was no reported H α emission, which is a common feature of such simple, low-intensity spikes².

Figure 1a shows a radio burst at 05:01:35 UT with an exceptionally slow frequency drift in the region 230–350 MHz. This is one of at least five events in the same frequency band with similar low drift rates. In addition, this flare had more typical type III and U bursts, as shown in Fig. 1b. In general, solar type III bursts at 325 MHz have an average drift rate of -150 MHz s^{-1} (ref. 3). The five slow drift bursts we saw with this flare had drift rates of -16 to -41 MHz s^{-1} . The peak fluxes range from 50 to $350 \times 10^4 \text{ Jy}$. Their polarization was $0 \pm 10\%$, which is identical to the associated regular type III bursts.

We have searched through the records of a previous study⁴ and found 34 more events with drift rates slower than -50 MHz s^{-1} , some unassociated with H α flares and the remainder from small events randomly distributed across the solar disk. Many more events of this type have probably been overlooked, being treated at the time as 'unusual phenomena'.

Solar type III bursts are caused by electron beams⁵. Their emission is through growing Langmuir waves of the bump-in-the-tail instability. The emission frequency is at the fundamental or second harmonic of these waves, which are close to the plasma frequency. The linear relation between emission frequency ν and the square of the electron density then leads to a simple relationship between the burst drift rate $d\nu/dt$ and the non-relativistic beam velocity v_B :

$$\frac{d\nu}{dt} = -\frac{\nu \cos \theta}{2\lambda} v_B \quad (1)$$

where λ is the density scale height of the atmosphere and θ is the angle between the beam motion and the density gradient. In a stationary isothermal atmosphere, λ is constant. For normal solar abundances and a temperature of $2 \times 10^6 \text{ K}$, which is reasonable for the low corona, λ is 10^5 km .

The slowly drifting event in Fig. 1a has a drift rate of $-19.6 \pm 1 \text{ MHz s}^{-1}$ at 310 MHz. Assuming $\cos \theta$ close to unity, equation (1) yields a beam velocity of $13,000 \text{ km s}^{-1}$, less than double the mean thermal electron speed in the low corona.

The low velocities derived for the slowly drifting bursts cannot be explained by the electron beams invoked for type III bursts for two reasons. (1) Landau damping inhibits the growth of Langmuir waves with velocities below about four times thermal. (2) The collision time for deflection (tending towards isotropy) of electrons having twice the thermal velocity is 0.6 s, assuming the radiation is at the second harmonic; that is, assuming a source density of $3 \times 10^8 \text{ cm}^{-3}$. For radiation at the fundamental frequency, the collision time is four times smaller. The lifetime of an electron beam with the observed velocity is therefore much shorter than the observed duration (up to 3.5 s) of the slowly drifting bursts.

A beam with $\cos \theta \ll 1$ and normal velocity (10^5 km s^{-1}) would require a nearly horizontal path of about half a solar radius. Such a magnetic structure is unlikely to exist at a density corresponding to the lower corona. Furthermore, the presence of normally drifting type III bursts (Fig. 1b) suggests that this possibility can be discarded; it is also evidence for a typical coronal density scale height such as we have adopted.

One way to increase the collisional lifetime would be to enhance the temperature. Unfortunately, however, this would also increase the scale height. The observed frequency range of $\sim 60 \text{ MHz}$ of the slowly drifting bursts requires a vertical dimension of 0.4λ . For 10^7 K , this amounts to $200,000 \text{ km}$ at a density of at least $3 \times 10^8 \text{ cm}^{-3}$. Since the radio emission started

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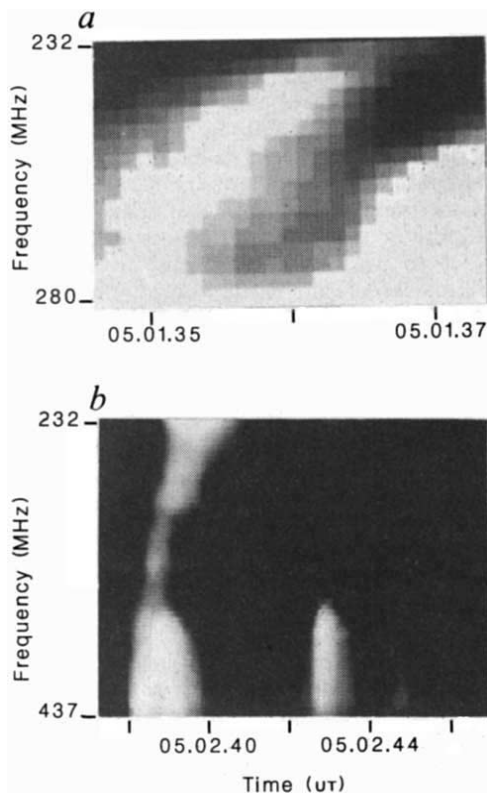


Fig. 1 *a*, The spectrogram (frequency versus time) of a slow-drift radio burst, probably followed by a similar event. For comparison, *b* displays regular type III bursts and a possible U-burst. The scales on the two axes are different from *a*, but the ratio of the scales is the same.

only 20 s after the soft X-ray enhancement, but 10 s before the hard X-rays, the growth of such a hot plasma column would have started long before the flare. There is no observational support for such an interpretation.

Shock waves in the corona have a low speed, and are frequently observed to excite type II radio emission. They have typical velocities of 600 km s^{-1} , and reach only $3,000 \text{ km s}^{-1}$ in exceptional cases⁶. Considering the fact that the observed slowly drifting events are relatively weak, with no sign of type-II-like substructure, a moving shock may be safely excluded as the source. Similarly, whistler and Alfvén wave solitons (invoked for the interpretation of intermediate-drift bursts) move at about the Alfvén velocity. The observed speeds and emission frequency would, however, require a plasma β of $\leq 10^{-4}$.

Among all known agents, the observed events can most plausibly be explained by a beam of protons. Proton beams in the energy region 10^2 – 10^3 keV have recently been proposed⁷ as the primary energy transfer mechanism between the energy release site, assumed to be in the corona, and the chromosphere, where the classical signatures of solar flares originate. This proposal was formulated to account for the appearance of nuclear-line γ -rays during the impulsive phase and strong plasma turbulence and upflows before the impulsive phase, neither of which can be explained satisfactorily by the traditional non-thermal electron model. The speeds derived from the radio features correspond to proton energies of 0.56–3.7 MeV. The lifetime of such protons in the source region is $>1 \text{ min}$. Proton beams are unstable to growing ion acoustic waves. The instability probably saturates before beam destruction, as in electron beams. Radio emission may be produced by coupling of the low-frequency waves to weak Langmuir waves of some other source⁸, an emission process that has been proposed for solar noise storms⁹.

Such a source could be the electron current which accompanies a freely-streaming proton beam (equivalent to the return current invoked in electron beam models to avoid charge separation).

We conclude that extremely slowly drifting type III bursts are probably signatures of proton beams at the Sun.

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1. Perrenoud, M. R. *Sol. Phys.* **81**, 197–203 (1982).
2. Simnett, G. M. & Dennis, B. R. *Proc. 19th int. Cosmic Ray Conf.* Paper SH 1.2–4 (1985).
3. Elgaroy, O. & Lyngstad, E. *Astr. Astrophys.* **16**, 1–12 (1972).
4. Benz, A. O. & Zlobec, P. *Astr. Astrophys.* **63**, 137–145 (1978).
5. Goldman, M. V. *Sol. Phys.* **89**, 403–442 (1983).
6. Robinson, R. D. *Sol. Phys.* **95**, 343–358 (1985).
7. Simnett, G. M. & Strong, K. T. *Astrophys. J.* **284**, 839 (1984).
8. Melrose, D. B. *Plasma Astrophysics Vol. 1* (Gordon and Breach, New York, 1980).
9. Benz, A. O. & Wentzel, D. G. *Astr. Astrophys.* **94**, 100–108 (1981).

Radio method of mapping large-scale coronal magnetic fields on active stars

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A number of nearby late-type stars, including the Sun, are known to be active at radio wavelengths. Disregarding mass-loss giant stars and novae (where the emission is probably of thermal origin)¹, we find that most radio stars are RSCVn binaries and UV Ceti-type flare stars². Estimated brightness temperatures $>10^9 \text{ K}$ suggest that the emission mechanisms are non-thermal, and in some cases coherent³. Non-thermal radio emission from active stars is often moderately circularly polarized. In this respect the emission is similar to solar radiation at metre to centimetre wavelengths and may similarly arise from the acceleration and subsequent trapping of mildly relativistic electrons in intense magnetic fields above star spots. I describe here a radio method which measures the longitudinal magnetic sector structure of the solar corona by observing the Sun 'as a star' and monitoring the sense of circular polarization of metre-wavelength emission as a function of rotational phase. The method is tested by comparing synoptic plots of solar radio-noise-storm polarities derived from high- and low-resolution observations over a period of four years at Culgoora. This method may be applied to the measurement of large-scale coronal fields on other active stars.

A recent study of observations with the Culgoora radioheliograph has shown that solar noise-storm polarities can be used to map the large-scale coronal magnetic sector structure⁴. Outlines of the yearly patterns are shown in Fig. 1*b*. Note the characteristic two- or four-sector structure in longitude, similar to that observed in the interplanetary medium⁵. A comparison of noise-storm patterns with the computed heliospheric current sheet obtained by extrapolating measured photospheric magnetic fields⁶ shows a one-to-one correspondence assuming o-mode radiation⁴.

The discovery of a longitudinal distribution in noise-storm polarities was unexpected, because noise storms had been assumed to be associated with the dominant sunspot polarity, which, as Hale⁷ first pointed out, is opposite in each solar hemisphere. Two explanations have been offered for this longitudinal effect⁴. One possibility is that the noise-storm polarity is not imposed at the source, but is a response to propagation conditions between the source and the observer. For instance, if the field direction, relative to the ray path, reverses between the source region and the high corona, then the ray must pass through a region of quasi-transverse propagation. If weak coupling applies in this region⁸ (that is, o-mode remains o-mode throughout the region), then the observed polarization of the

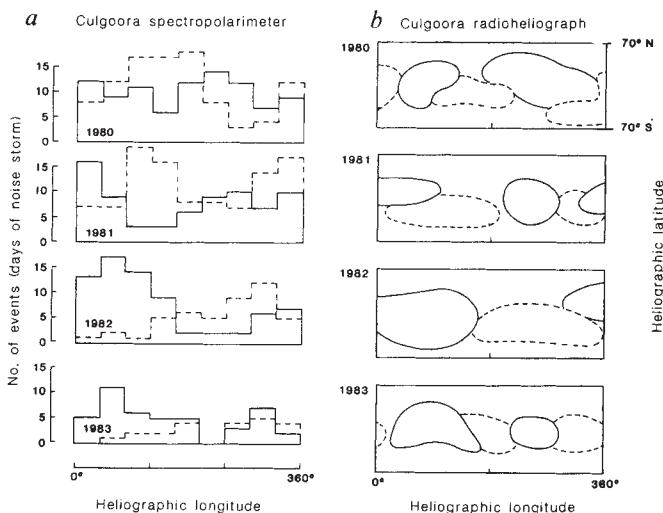


Fig. 1 *a*, Yearly Carrington plots of the noise-storm polarity for the Sun during 1980-83, derived assuming that the emission occurs from the central meridian. *b*, The true two-dimensional distribution of polarized noise storms on the Sun during 1980-83. Solid lines refer to left-handed and dashed lines to right-handed circular polarizations.

noise storm will be determined by the polarity of the high coronal or interplanetary magnetic field. On the other hand, if strong coupling applies, then the polarization will be maintained in its original sense and will reflect the dominant polarity of the sunspot group. The close positional agreement between noise-storm polarity and sunspot field led Stewart⁴ to favour the second explanation of strong coupling.

Such a distinct longitudinal magnetic structure, as shown in Fig. 1*b*, suggests that monitoring the Sun 'as a star' may also reveal such a pattern. Figure 1*a* shows Culgoora spectropolarimeter (single-dish) observations of noise-storm polarities over the same period. The yearly synoptic plots of Fig. 1*a* were derived as follows. First it was assumed that on each day the source occurs at the solar longitude of central meridian. (On the few days when there was more than one noise-storm source active on the Sun the data were discarded.) Then the data were averaged over 40° of solar longitude (3 days) and over 13 successive solar rotations (1 year) to improve the statistics. The justification for averaging the data is as follows. First, because of the low directivity of noise storms, ~30° to half-power points⁹, the noise storm may be observed for several days away from central meridian passage. Second, at times away from solar maximum the large-scale magnetic field patterns remain fairly stationary for periods of ~1 yr. Comparison of Fig. 1*a* with true longitudinal distribution of noise-storm polarity derived by two-dimensional radioheliograph observations (Fig. 1*b*) shows that the 'Sun as a star' method detects most magnetic sectors.

Could this method work on the Sun for high frequencies? As yet no statistical tests have been done. One might argue that, because the coronal source region at higher frequencies is closer to the surface of the Sun, the magnetic fields will be more complex because of more closed-loop configurations, and that consequently no net polarity effects will be observed. However, it is certainly true that individual events such as the slowly varying component and type IV flare bursts at microwave frequencies exhibit moderate degrees of circular polarization. Furthermore, these emissions are associated with sunspot active regions, as are noise storms at lower frequencies. Thus, the argument that noise storms, and other forms of solar radio emission which are associated with closed magnetic loop

configurations, should show a net zero degree of circular polarization because of the bipolar nature of the source region is not substantiated by observations.

Turning now to other stars which are active at radio wavelengths, we note that Gibson¹⁰ has proposed a leading-trailing starspot magnetic field model for RSCVn binaries. He argues that the more highly polarized binaries have orbits which are tilted with respect to our line of sight; that is, they are observed more or less 'pole-on', whereas the systems viewed 'equator-on' do not exhibit significant circular polarization. According to Gibson's model one would not expect to see a net polarization for the latter systems because the number of active regions in the Northern Hemisphere should equal the number in the Southern Hemisphere. This is precisely the situation for noise storms on the Sun, but, as shown in Fig. 1, we do see a net polarity and furthermore this polarity reverses as the Sun rotates. For these reasons it would seem worthwhile to monitor accurately the sense of circular polarization of RSCVn stars as a function of rotation phase over many cycles, to see if there are any systematic variations with stellar longitude. Observations to date on two well-known RSCVn binaries, HR1099 and UX Ari, suggest that the radio polarity reverses between 1.4 and 5 GHz but does not change with time¹¹. Both active stars are observed substantially pole-on, which may account for the absence of a rotational effect.

A number of solar-type stars, of spectral type G and other late types F and K, are known from optical and soft-X-ray observations to have active chromospheres and coronae. Yet apart from the GO V star χ_1 Ori (ref. 12), no others have been detected with the Very Large Array at centimetre wavelengths, and even the χ_1 Ori result may have been due to an outburst on the dwarf companion². It seems unlikely that the 17 candidates studied so far were all in a minimum activity phase when observed. Hence it seems that before stellar magnetic field mapping can be achieved by the above method at centimetre wavelengths, the sensitivity limits for radio detection need to be improved by using longer integration times, wider continuum bandwidths or larger collecting areas. From the solar analogue, we know that the radio emission often increases towards lower frequencies, so that it might be more profitable to concentrate on radio mapping at decimetre or metre wavelengths.

Of the 100 or so known radio stars, about half are dMe flare stars. Not much is known about the physical conditions in the radio source regions on these stars, but it is clear that the radio properties are very similar to those for solar flares. For this reason alone it would be worth putting considerable effort into a programme for systematically monitoring the circular polarization of selected flare stars with known rotation rates. Highly polarized radio emission lasting several days, without flare-like increases in intensity, has been observed recently from two late-type dwarf stars, UV Cet and YZ CMi (ref. 13). Such emission has been likened to solar noise-storm activity; if this were so, the method outlined above could reveal any large-scale magnetic field structures present in the coronae of such stars.

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1. Wright, A. E. in *Proc. 2nd Asian-Pacific Regional IAU Meeting on Astronomy* (eds Hidayat, B. & Feast, M. W.) 61-73 (Tira Pustaka, Jakarta, 1981).
2. Pallavicini, R., Willson, R. F. & Lang, K. R. *Astr. Astrophys.* **149**, 95-101 (1985).
3. Dulk, G. A. A. *Rev. Astr. Astrophys.* **23**, 169-224 (1985).
4. Stewart, R. T. *Sol. Phys.* **96**, 381-395 (1985).
5. Svalgaard, L. & Wilcox, J. M. A. *Rev. Astr. Astrophys.* **16**, 429-443 (1978).
6. Hoeksema, J. T., Wilcox, J. M. & Scherrer, P. H. *J. geophys. Res.* **88**, 9910-9918 (1983).
7. Hale, G. E. *Astrophys. J.* **38**, 27-98 (1913).
8. Cohen, M. R. *Astrophys. J.* **131**, 664-680 (1960).
9. Steinberg, J. L., Caroubalos, C. & Bougeret, J. L. *Astr. Astrophys.* **37**, 109-115 (1974).
10. Gibson, D. M. *Proc. 3rd Cambridge Workshop, Cool Stars, Stellar Systems and the Sun* (eds Balinas, S. L. & Hartmann, L.) 197-201 (Springer, Berlin, 1983).
11. Mutel, R. L., Lestrade, J.-F. & Doiron, D. J. in *Radio Stars* (eds Hjellming, R. M. & Gibson, D. M.) 259-265 (Reidel, Dordrecht, 1985).
12. Gary, D. E. & Linsky, J. L. *Astrophys. J.* **250**, 284-292 (1981).
13. Kundu, M. R. & Shevgaonkar, R. K. *Astrophys. J.* **297**, 644-648 (1985).

Chaotic rotation of Hyperion?

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Saturn's satellite Hyperion has been predicted to be in a chaotic rotation state as a result of its irregular shape and tidal interactions¹. However, an analysis of Voyager 2 images by Thomas *et al.*² contradicted this prediction. These authors analysed 14 images obtained over 61 days and interpreted them to be consistent with a coherent (non-chaotic) rotation period of 13.1 days. The interpretation of the Voyager data has, however, been criticized by Peale and Wisdom³, who argued that the low sampling frequency does not allow chaotic or non-chaotic rotation to be distinguished. We report here new observations which were obtained with a higher sampling frequency. These data conclusively show that the 13.1-day period found by Thomas *et al.* was not due to coherent rotation.

Charge-coupled device (CCD) images of Hyperion were obtained on eight nights between 16 and 30 April, 1985 UT using the McDonald Observatory 0.76-m telescope and a CCD camera with an RCA 320 × 512 detector. Hyperion was easily identified using offsets from the 1985 *Astronomical Almanac*. Two to six images were obtained each night through a V filter and exposure times ranged from 20 to 60 s. For each image, Hyperion was placed near the edge (20–30 pixels) of the chip so that almost all of the glare from Saturn was excluded. A nearby comparison star, SAO159426, spectral type K2, was imaged separately in the same area of the chip. V magnitude calibrations were performed by imaging solar-type standard stars⁴.

Photometric measurements were performed by integrating each Hyperion image within a circular aperture. Aperture sizes were chosen by measuring the width of Hyperion's image profile out to the 99% level. Sky subtraction was performed using an annulus of equal area around the Hyperion aperture. All images were flat-fielded and bias-corrected. Hyperion's light curve is presented in Fig. 1. The comparison star, SAO159426, was reduced in an identical fashion and remained at a constant V magnitude of 9.44 ± 0.02 .

Our light curve of Hyperion (Fig. 1) shows an amplitude of nearly 1 magnitude. The data cover a span of 14 days and rule out a 13.1-day period because the 16 and 17 April data are near maximum light and the 29 and 30 April data are near minimum light. These new observations imply that the 13.1-day period derived from the Voyager data² was not due to coherent rotation.

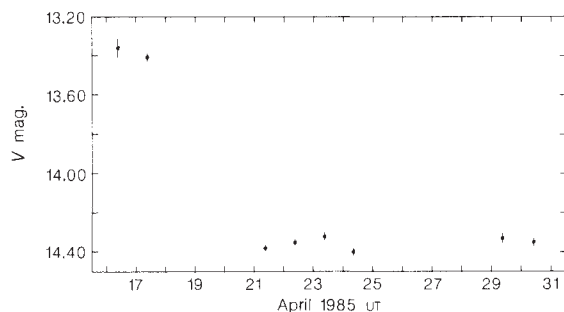


Fig. 1 Light curve of Hyperion obtained in April 1985. The dates (UT) of the observations are given on the abscissa. The V magnitudes from each night have been reduced to a common viewing geometry: $r = 9.5$ AU, $\Delta = 8.5$ AU, phase angle = 2.5° , where r = Sun–Saturn distance, Δ = Earth–Saturn distance. This latter value represents the mean of the range of observed phase angles (1.8 – 3.2°) and the phase correction used a coefficient of 0.026 mag. deg^{-1} . The error bars indicate the photometric accuracy for the mean of the images on each night, which was typically 0.04 mag. or better.

Thus, the contention³ that the Voyager period could have resulted from the infrequent sampling of a chaotic rotation is supported.

This new result alone does not resolve the question as to whether Hyperion is in a chaotic or non-chaotic rotation state. If Hyperion's rotation is non-chaotic, the light-curve periods determined at various times should always be in close agreement. The Voyager light curve obtained over a 61-day interval in 1981 displayed several maxima and minima, indicating that if Hyperion is in a non-chaotic state, its rotation period is a small fraction of 61 days. Thomas *et al.* performed a period search over the range of residuals which tightly constrains their solution to a value near 13.1 days.

If Hyperion is in a chaotic rotation state, the light-curve period would be changing randomly with time. Light-curve periods determined at various times would not be expected to be in close agreement. Therefore, the incompatibility of our new data with the period found by Thomas *et al.* is fully consistent with Hyperion being in a chaotic rotation state. Although this incompatibility does not conclusively prove that Hyperion's rotation state is chaotic, it is strong evidence in favour of this hypothesis.

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Received 20 November 1985; accepted 6 January 1986.

1. Wisdom, J., Peale, S. & Mignard, F. *Icarus* **58**, 137–152 (1984).
2. Thomas, P., Veverka, J., Wenkert, D., Danielson, G. E. & Davies, M. E. *Nature* **307**, 716–717 (1984).
3. Peale, S. & Wisdom, J. *Bull. Am. astr. Soc.* **16**, 686 (1984).
4. Landolt, A. U. *Astr. J.* **78**, 959–981 (1973).

Experimental observation of ultra-low-frequency waves generated in the ionosphere

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Nonlinear properties of the ionosphere can be used to produce electromagnetic waves of ultra-low frequencies (ULF, 0–300 Hz). This can be achieved either by modulating one strong high-frequency (HF, 3–30 MHz) wave or by beating two strong radio-waves with a frequency difference Δf which lies in the ULF band. I report here observations of such ULF signals in two experiments. In the first, four HF transmitters were split into two pairs and operated with a frequency separation Δf between each of the pairs. In the second experiment, all of the transmitters were operated at the same high frequency and were pulsed. The ULF signals at 3, 5 and 6.25 Hz and with intensities of ~ 150 – 350×10^{-11} G $\text{Hz}^{-1/2}$ were received on the island of Mona, which is ~ 150 km west of the transmitting site.

Periodic plasma heating has been used for ULF wave generation in the ionosphere^{1–3}. These results have typically been explained in terms of changes in conductivity and subsequent modulation of the ambient current system in the lower ionosphere. A different experiment is presented here, in which two HF transmitters were used with a frequency separation of Δf . The beat frequency with proper w and k matching conditions was produced in the night-time F-region of the ionosphere. The mechanism does not require any existing current system in the ionosphere. The k matching conditions are different from those obtained by periodic plasma heating. The nonlinearity in this case arises through ponderomotive forces^{5–7}. In the three-wave

coupling process, the mixing of the two pump signals gives rise to the excitation of wave modes with frequencies and k -vectors that are the difference and/or sum of those of the injected signals. This has been considered by Papadopoulos⁸ *et al.*, who also predict that the threshold for the absolute instability is lowered as the Δf is decreased. I have made preliminary observations of such ULF waves, at Arecibo during a preliminary experiment in 1984. A more detailed campaign has recently been performed using the facilities under the control of the Arecibo Observatory in Puerto Rico.

The essentials of the experiment are summarized in Table 1.

The receiving system that was built around the sensitive magnetometer systems provides extremely low noise and has a flat response for magnetic field in the frequency band 0.001–500 Hz. The three-axis magnetometer system was employed both at Los Canos (7 km from the HF transmitters at Islote) and at the small island of Mona (150 km west of Islote).

The Islote transmitters were operated at both 5.1 and 3.1 MHz. Two different experiments were performed. In one, the four HF transmitters were split into two pairs and a small frequency difference was introduced between each pair of transmitters. Nonlinear mixing in the ionosphere produced the beat (difference) frequency. The beat frequency was varied only on the half-hour or hour and was cycled between 3 and 5 Hz.

Two examples of the data are shown in Figs 1 and 2. The HF transmitters were operating at 5.1 MHz and 5.1 MHz + 5 Hz during 16:30–17:30 AST. The spectrum of the 0–10-Hz signal received in this time interval is shown in Fig. 1, and Fig. 2 shows the spectrum for the next half-hour. During this period (17:30–18:00 AST) the difference frequency was changed from 5 to 3 Hz. The received spectra clearly indicate the 5 or 3 Hz signals. The signal-to-noise ratio is ~ 1 or 2.5 and the absolute magnitude of these signals is ~ 150 or $350 \times 10^{-11} \text{ G Hz}^{-1/2}$.

All three components of the field were monitored during these experiments. Significantly, however, the signals were received only in the coil which was aligned in the east/west direction. There was no detectable signal in either the north/south or vertical component of the magnetic field. The coherent nature of the signal can be used to improve the signal-to-noise ratio. Using a narrower (fast Fourier transform analysis) filter the signal-to-noise ratio can be greatly improved. However, the signal was found to fade considerably. The fading rate determined the narrowest filtering that could be used. Using a 5-mHz filter, the signal-to-noise ratio was improved to about 20. Examples of such spectra are shown in Fig. 3 and the temporal history of the 5.0-Hz signal analysed in 40-s bins is shown in Fig. 4.

In the second experiment, all four transmitters at Islote were turned on and off at ULF rates of 5.0 and 6.25 Hz. In general, there was good coincidence of the transmitter operation and ULF detection. The duration of this experiment was too small for any statistics to be compiled. Nevertheless, ULF signals of either beat or modulation origin were positively identified at Mona and, on occasion, at Los Canos near the Arecibo Observatory. The sites near the Arecibo Observatory have a higher noise background level in the ULF band (civilization or cultural noise) than those in Mona. The experimental observations can be summarized as follows:

(1) ULF signals were observed on Mona and at Islote at frequencies of 3 and 5 Hz when the HF transmitters were operated with these different frequencies and the ionospheric conditions were appropriate.

(2) ULF signals were detected only in the east/west component of the magnetic field. The average values of these signals were typically $150\text{--}350 \times 10^{-11} \text{ G Hz}^{-1/2}$.

(3) Large signals occurred in bursts, as short as 40 s (limited by our present analysis). The signal-to-noise ratio was as large as 20.

(4) The occurrence frequency for such short bursts when the signal-to-noise was >1 (see Fig. 4) was $\sim 50\%$. This does not

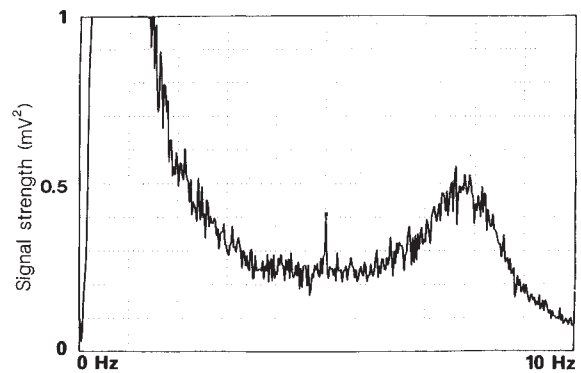


Fig. 1 Spectrum of the received signal in the 0–10 Hz band. Signal strength in mV^2 corresponds to $1.7 \times 10^{-17} \text{ G}^2 \text{ Hz}^{-1}$, full scale (14 February 1985). The receiver was located at Mona Island. The data cover the period 16:30–17:30 AST. The HF transmitters were operating at 5.1 MHz and with a difference frequency $\Delta f = 5$ Hz during this period. The cursor is located at 5.0 Hz. The magnitude of the 5.0-Hz signal is $\sim 160 \times 10^{-11} \text{ G Hz}^{-1/2}$.

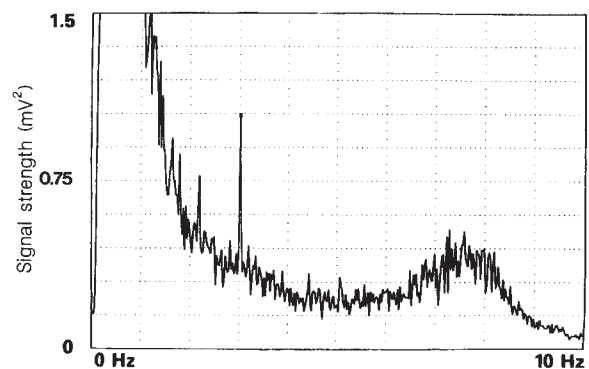


Fig. 2 Spectrum of the received signal (similar to Fig. 1) averaged over the period 17:30–18:00 AST. The difference frequency was 3.0 Hz. The vertical scale corresponds to $2.6 \times 10^{-17} \text{ G}^2 \text{ Hz}^{-1}$. The magnitude of the 3.0-Hz signal is $\sim 340 \times 10^{-11} \text{ G Hz}^{-1/2}$.

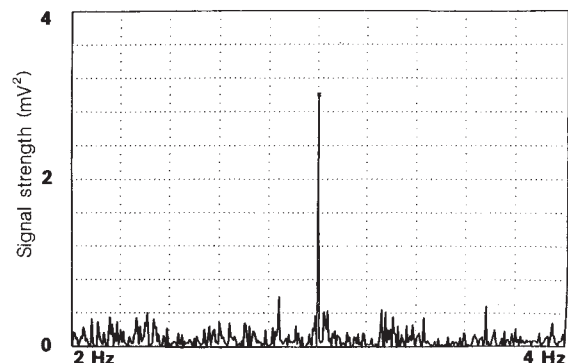


Fig. 3 Spectrum for 14 February 1985 during 17:37–17:40 AST. The spectral bandwidth is 5 mHz. Note the large signal-to-noise ratio.

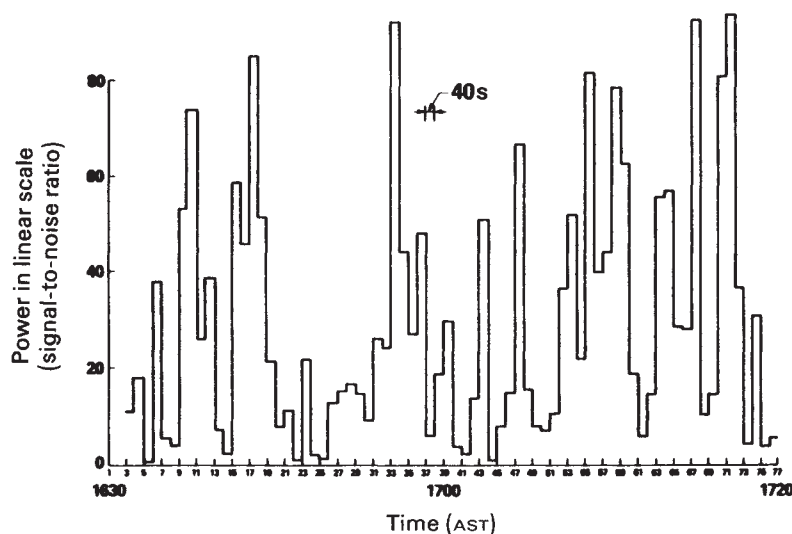


Fig. 4 Short-term (40 s) fluctuations in 5.0-Hz signals during 16:30–17:27 AST on 14 February 1985. Note the large variability in the signal-to-noise ratio.

include the period when the ionospheric conditions were unsuitable.

(5) When transmitters were pulsed on and off at 5.0 and 6.25 Hz, signals were detected at the respective frequencies; that is, 5.0 and 6.25 Hz.

Although it is too early to specify the exact nature of the source mechanism for ULF signals, some preliminary deductions can be made from the Arecibo observations. The beat frequency experiments presented here are different from the low-frequency wave excitation by periodic plasma heating produced by modulation in conductivity and do not require an existing current system in the ionosphere. Indirect evidence of such three-wave mixing has been obtained in another experiment⁷ in which, under the influence of two strong HF waves, the ionospheric reflected signals contained sidebands which were multiples of the difference (Δf) between the two pumping frequencies.

The Arecibo heater has a beamwidth of about 10° . The ray at the edge of the heater thus enters the horizontally stratified ionosphere at an angle of 5° . During the process of reflection, significant bending in the horizontal direction can take place. Interactions of the radio waves with the ionized plasma occur along the ray path⁸. The size of the interaction region depends on the extent of the horizontally propagating rays. These horizontal propagations could be significantly increased, depending on the exact nature of the ionospheric profile dN/dh ; (gradient of electron density with height). The smaller the gradient, the larger the horizontal propagation. Ionospheric tilts, waves, irregularities and possible ducting could also affect these propagations.

In spite of the limited statistics, it can be surmised that the large signal-to-noise ratio of ~ 20 may not be generated from the 'classical nonlinear theories', for example, electron heating, two-frequency beat, or current modulation. The sporadic nature of these signals strongly indicates that some instability process is operative. With our limited transmitted power we may have attained the threshold for 'absolute instability' only for certain ionospheric situations. Also the presence of gravity waves and other irregularities could have favoured such situations for brief periods and aided in the production of ULF waves.

The predominance of the east/west component of the magnetic field over the north/south and vertical components during the beat experiment indicates that the nonlinear current is in the horizontal direction and flowing in the magnetic meridian plane. In the F-region, where the field-aligned Pederson conductivity is dominant, most of the currents are forced to flow in the north/south direction.

Periodic plasma heating has been used for ULF wave generation in the ionosphere^{1–3} and there are several physical

Table 1 Experimental arrangements for generation and detection of ULF signals

HF transmitters

Location	Islote, Puerto Rico
Typical operating power	4×100 kW fed into an antenna array of 22–25 dB gain
Operating frequencies	3.175 and 5.1 MHz
Operating mode	(1) Pulsed at ULF range of 1–10 Hz (2) Beat frequency generation in the ULF range of 1–10 Hz.
Duration of experiment	Four periods of 8 h each during 31 January–15 February 1985

Receiving systems

Sensors	Magnetic loops (3 axes) Sensitivity, 500×10^{-5} mV G ⁻¹ Noise level, 10^{-9} G Hz ^{-1/2}
Locations	Mona Island, ~ 150 km west of Islote Los Canos, 7 km south-west of Islote Near the Arecibo Observatory, ~ 20 km south-west of Islote

processes that could be invoked for electromagnetic wave generation induced by periodic heating. Further observations of ULF signals in pulsed conditions of various durations are necessary to establish the validity of any one theory.

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- Getmanster, G. G. *et al. Izv. vuzov. Radiofizika* **26**, 1017 (1977).
- Stubbe, P. *et al. J. atmos. terr. Phys.* **44**, 1025–1041 (1982).
- Ferraro, A. J. *et al. J. atmos. terr. Phys.* **44**, 1113 (1982).
- Tsytovich, V. N. *Non-linear Effects in a Plasma* (Plenum, New York, 1970).
- Fejer, J. *Rev. Geophys. Space Phys.* **17**, 135–153 (1979).
- Lavergnat, J., Bauer, P., Delahaye, J. Y. & Ney, R. *Geophys. Res. Lett.* **4**, 417 (1977).
- Ganguly, S. & Gordon, W. E. *Non-Linear Mixing Processes in the Ionosphere. Pap. presented at URSI General Conf. Florence* (1984).
- Papadopoulos, K., Ko, K. & Tripathi, V. *Phys. Rev. Lett.*, **51**, 463 (1983).
- Papadopoulos, K. & Chang, C. L. *Geophys. Res. Lett.* (in the press).

Vertical distribution of dimethylsulphide in the marine atmosphere

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Dimethylsulphide (DMS) is excreted into sea water by marine phytoplankton and then transferred across the air/sea interface into the atmospheric boundary layer¹⁻⁵. This process represents about one-half of the world's estimated natural sulphur emissions⁶, which are comparable in magnitude to the SO₂ emissions from fossil-fuel burning⁷. The distribution and chemical reactions of DMS in the marine surface boundary layer have been described in detail elsewhere⁸. Here we present the first data on the vertical distribution of DMS in the marine troposphere. Our observations agree well with the distributions predicted by a two-dimensional model calculation which includes convective transport and chemical processes. This agreement supports the validity of our previous estimate of $\sim 40 \text{ Tg S yr}^{-1}$ for the input of DMS from the oceans to the atmosphere and suggests that the oxidation of DMS can contribute significantly to the SO₂ levels observed in the free troposphere.

Samples were collected off the coast of Barbados in the tropical Atlantic/Caribbean region (12–14° N, 56–59° W) during the NASA Global Tropospheric Experiment/Atmospheric Boundary Layer Experiment (GTE/ABLE) Barbados Mission (13–30 June 1984). The flights we discuss here took place on 18–27 June 1984, and sampled the marine troposphere between 150 and 5,300 m altitude at four to six altitude levels per flight. On 19 June, samples were collected at ground level at Ragged Point, the easternmost point of Barbados.

DMS was sampled using a 5-mm (internal diameter) stainless steel intake and the gold-surface pre-concentration technique described in detail previously^{8,9}. Air volumes were measured by an integrating mass flowmeter (Teledyne Hastings-Raydist, Hampton, Virginia); the sample volumes ranged between 7 and 70 litres. The samples were analysed within a few hours after the flight by thermal desorption from the gold surface, followed by gas chromatography with flame photometric detection⁹. DMS is stable on the gold surface without detectable change for at least 18 h.

The results of the DMS measurements are shown in Fig. 1. Three of the flights (4, 5 and 7; Fig. 1a) took place under stable, 'undisturbed' conditions; that is, in the absence of convection by precipitating cumulus clouds. Flight 4 took place in essentially cloudless conditions, whereas during flights 5 and 7 fair-weather (trade-wind) cumulus was present below the trade-wind inversion, covering about one-tenth of the sky. The heights of cloud bases and tops are indicated in Fig. 1a (cloud symbol). Flights 6 and 8 took place in the presence of large cumulus congestus clouds with tops above 6 km (convective, 'disturbed' conditions). Samples were collected both upwind and downwind of large cumulus clouds (Fig. 1b).

The distribution of DMS in the troposphere was quite consistent for the flights in undisturbed conditions. In the lowest few hundred metres, the concentrations were 40, 90 and 102 p.p.t.v. (parts per 10¹² by volume) for flights 4, 5 and 7, respectively. This range of values agrees well with four ground-level measurements made on 19 June at Ragged Point, Barbados (70 ± 6 p.p.t.v. DMS). Since these values are also very close to

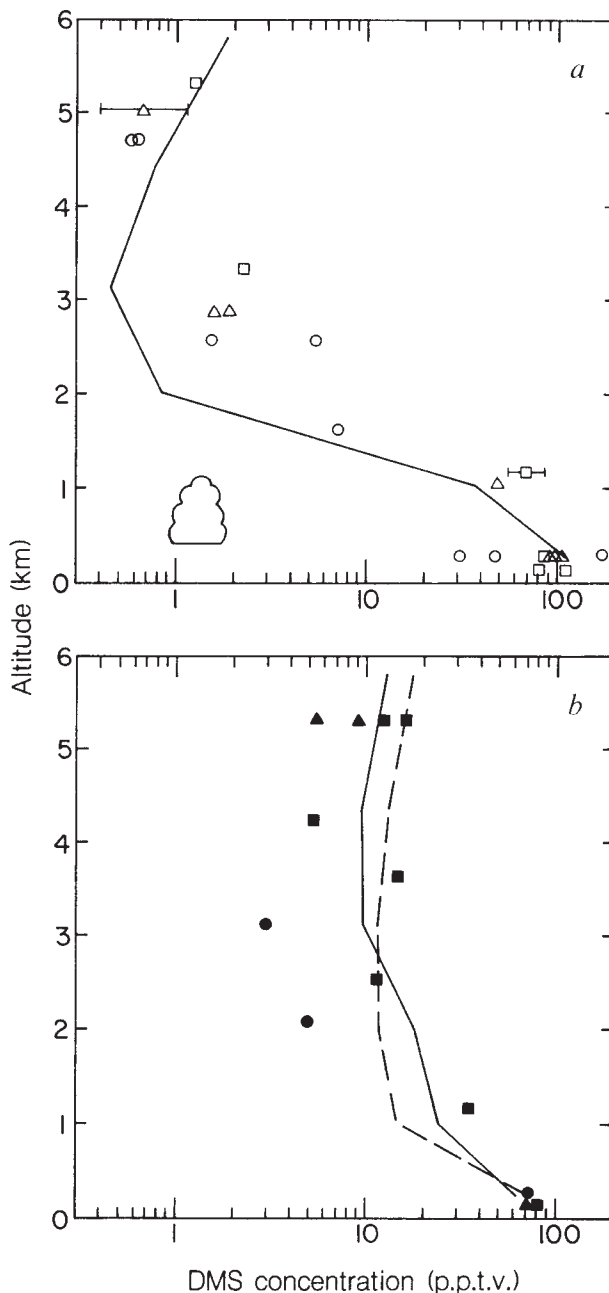


Fig. 1 Comparison of observed DMS concentrations in the tropical marine atmosphere (symbols) with values predicted by the 'Staubsauer' model¹² for stable (a) and convective (b) conditions. All model results are 24-h averages. a, The model simulation represents tropical regions with widely scattered fair-weather clouds. Data are from flight 4 (○), which took place under practically cloudless conditions, and from flights 5 (□) and 7 (△), during which fair-weather (trade-wind) cumulus was present. On flight 7, samples were collected between 00:15 and 04:00 local time. The cloud symbol indicates the heights of cloud bases and tops. An indication of the analytical uncertainty is given for one high and one low data point. b, The model simulations are for tropical areas experiencing intermittent convection (solid line) and synoptic convective disturbances (broken line). Data are from measurements made during a convective episode upwind (●, flight 6) and downwind (■, flight 6; ▲, flight 8) of a large cumulus congestus cloud.

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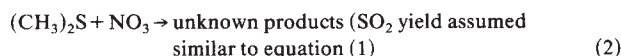
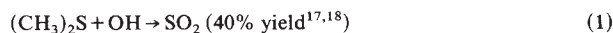
our previous measurements of DMS in the tropical marine boundary layer over the Atlantic and Pacific Oceans⁸, we assume that the conditions during the Barbados mission were reasonably representative of the tropical marine atmosphere. The concentration decreased with increasing altitude, first gradually to the level of the trade-wind cloud tops, then more steeply, to values of a few p.p.t.v. above the trade-wind inversion (2–3 km). This pattern was observed during both the daytime flights (4 and 5) and the night-time flight (7). Under disturbed conditions (flights 6 and 8), when the trade-wind inversion was absent, the DMS concentrations at >2 km altitude were about an order of magnitude higher than under undisturbed conditions; they were also higher downwind of the cumulus than upwind (Fig. 1b). In the boundary layer, DMS levels (81 and 77 p.p.t.v.) were similar to those present under undisturbed conditions. This increase in the DMS levels in the free troposphere indicates increased upward transport of DMS under highly convective conditions. The night-time DMS concentration in the boundary layer was higher than the daytime concentrations: 102 as against 70 p.p.t.v., the average of the daytime measurements. Although this single measurement cannot by itself be considered evidence for diurnal variability, a night/day ratio of 1.45 is consistent with the diurnal cycles in DMS concentration we have observed previously in extended time series⁸.

Due to its relatively rapid reaction with the OH radical, DMS has been thought to be of importance for the production of SO₂ and excess (non-seasalt) sulphate only in the lowest one or two kilometres of the marine atmosphere^{10,11}. Recent models^{12,13} suggest, however, that DMS can be intermittently vented to the free troposphere by the action of convective clouds. The 'Staubsauger' model¹² combines a photochemical model with a two-dimensional description of convective vertical transport within larger synoptic circulations, representing the characteristics of atmospheric circulation within the tropical regions. It includes the effects of both rapid vertical transport by large convective cloud systems, which can transport reactive species into the free troposphere before they are oxidized by photochemical reactions, and the slower, synoptic-scale circulation outward and downward from these storm systems. The model gives a continuous representation of trace-gas vertical distribution from highly convective regions to stably stratified regions of subsiding air remote from convective areas, encompassing the range of meteorological situations found during the Barbados mission. In the following sections we compare the predictions obtained from the Staubsauger model with our observations from the Barbados flight experiment.

In order to obtain a realistic estimate of the sea-to-air flux of DMS for input to our model, we applied the 'stagnant-film' model of Broecker and Peng¹⁴, using the equation $F = (D/z)C_1$, where F is the sea-to-air flux of DMS, D the DMS diffusion coefficient in sea water ($1.2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 26 °C, estimated according to ref. 15), z the 'stagnant-film thickness' (50 µm, mean of tropical North Atlantic values from ref. 16), and C_1 the DMS concentration in surface sea water. The surface wind speeds upwind of the study area were consistently in the range 10–20 knot. In view of the uncertainty in the relationship between the film thickness and wind speed and the relatively small variations in wind speed during the study period, it appears justified to apply a single film-thickness value to the flux estimate for all flights. Since no surface water DMS measurements were available for the time of the Barbados experiment, we used the mean of our data from the tropical Atlantic, which were collected on three cruises in 1980, 1981 and 1983 (83 ng S(DMS) m⁻³, 147 samples; ref. 5 and M.O.A., unpublished data). From these data, we predict a flux of 170 µg S(DMS) m⁻² day⁻¹ (0.062 g m⁻² yr⁻¹). The uncertainty of this estimate is of the order of a factor of two, largely due to the uncertainty inherent in the stagnant-film model.

The reactions of DMS with OH and NO₃ radicals were simulated as they respond to the diurnally varying photochemistry

of the troposphere. The significant reactions which consume DMS are:



OH is produced photochemically and consumes DMS during the day, whereas NO₃ is destroyed photochemically and can only limit the build-up of DMS at night. We used reaction rate expressions from Baulch *et al.*¹⁹; the other model inputs and photolysis rates were as in ref. 12. Baulch *et al.*¹⁹ suggest a rate for the reaction of OH with DMS of $5.5 \times 10^{-12} \exp(179/T) \text{ molecule}^{-1} \text{ cm}^3 \text{ s}^{-1}$. Atkinson *et al.*²⁰ suggest a rate of consumption of the reactants NO₃ and DMS of $\sim 5 \times 10^{-13} \text{ molecule}^{-1} \text{ cm}^3 \text{ s}^{-1}$, but uncertainties described in ref. 21 allow rates up to 9×10^{-13} . NO₃ measurements from Barbados are not available; therefore we have to assume that the concentrations during our flights were similar to those observed over the Pacific by Noxon²² (~ 0.3 p.p.t.v.). We included in our simulation a reaction rate expression recently proposed by Johnston *et al.*²³ for the unimolecular decomposition of NO₃, and obtained NO₃ values of ~ 0.1 – 0.2 p.p.t.v. at night. At these concentrations, NO₃ consumes little of the DMS in our reaction scheme, in agreement with the calculations of Winer *et al.*²⁴, who found that 0.3 p.p.t.v. NO₃ had little effect on the predicted concentrations of DMS. The NO_x field used for the model has been selected in such a way as to provide an ozone field typical of tropical marine conditions⁷; the resulting model ozone field agrees well with ozone measurements made during the Barbados experiment (G. L. Gregory, personal communication).

As the model curves in Fig. 1 show, there is good agreement between our observations and the model simulations of chemistry and transport. This agreement suggests that the input flux assumed in the model calculations (0.062 g S(DMS) m⁻² yr⁻¹) is indeed a realistic estimate for the DMS input to the atmosphere in this region, and supports our previous estimate of 40 Tg S yr⁻¹ for the global oceanic DMS source⁵.

Both the model results and the observations suggest a large variability in DMS concentrations in the atmosphere above 2 km, and a large difference between fair-weather and convective conditions. Most previous simulations of sulphur in the remote atmosphere have used the eddy-diffusion description of transport. Logan *et al.*¹⁰ used a one-dimensional model which produced results similar to ours when little or no convective activity is assumed (Fig. 1a); Rodhe and Isaksen's¹¹ two-dimensional model predicted similar vertical distributions for H₂S, the compound they used to illustrate the behaviour of a reactive reduced sulphur gas. Since eddy-diffusion calculations predict low DMS (or H₂S) concentrations throughout the middle and upper troposphere, only very slowly reacting sulphur species, for example COS¹⁰, were thought to contribute to the natural atmospheric sulphur cycle in the global troposphere beyond the first 1–2 km. In view of the relatively small source flux of COS⁶, the origin of SO₂ and sulphate throughout the troposphere^{25,26} could not be adequately explained. Our observations and model results, on the other hand, show that significant amounts of DMS exist in the middle troposphere in the presence of convective activity (Fig. 1b), suggesting that intermittent deep vertical transport of DMS may be responsible for a significant fraction of tropospheric SO₂. Depending on the assumed intensity of convection and the rate of SO₂ removal by heterogeneous processes in clouds, the model predicts between 20 and 65 p.p.t.v. SO₂ at 6 km, about half of the concentration actually observed in the free marine troposphere (85 ± 28 p.p.t.v.)²⁵.

Substantial questions remain about the meteorology and chemistry of DMS transport. As mentioned above, the relative yields of SO₂ and other products from DMS are not well established. Oxidants of DMS other than OH and NO₃ cannot be

excluded. What fragmentary evidence exists regarding the oxidation of DMS suggests that it may produce as intermediates moderately reactive compounds^{17,18}, which are oxidized further on timescales of several days or more and which may be very efficient in redistributing sulphur in the troposphere.

Furthermore, the meteorology of vertical transport needs further study. It is clear that some material from the boundary layer must be redistributed even to the top of the troposphere¹², but the global climatology of cloud incidence and the most typical vertical transport patterns of air parcels containing recent inputs of sulphur gases are still subject to debate^{12,13,17,18}.

Larger-scale motions of the atmosphere also play an important role in determining sulphur species concentrations. For example, Fig. 1b contrasts two simulations of a synoptic region (approximately 10° latitude by 10° longitude), each with the same cloud activity within the region. The profile with higher concentrations above 3 km (dashed line in Fig. 1b) simulates the effects of large-scale horizontal motions bringing in DMS from convective regions outside the model area; the other simulates a region with the same local meteorology in the absence of such advective transport (the winds in this case may be low, or they may blow from fair-weather regions or continents). The fact that the model tends to predict somewhat higher DMS concentrations above 3 km than are actually observed (especially during slightly convective conditions; Fig. 1a) may be due to such effects of large-scale meteorology. The model simulates the concentrations of DMS at high altitudes over an extended ocean, such as the Pacific, whereas the Atlantic Ocean near Barbados provides a shorter fetch (~4,500 km), and therefore less time to introduce DMS into the middle troposphere. The discrepancy found between simulations and observations at these altitudes is thus to be expected.

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- Vairavamurthy, A., Andreae, M. O. & Iverson, R. L. *Limnol. Oceanogr.* **30**, 59–70 (1985).
- Andreae, M. O. & Barnard, W. R. *Mar. Chem.* **14**, 267–279 (1984).
- Andreae, M. O. in *Biogeochemistry of Ancient and Modern Environments* (eds Trudinger, P. A., Walter, M. R. & Ralph, B. J.) 253–259 (Springer, Berlin, 1980).
- Cline, J. D. & Bates, T. S. *Geophys. Res. Lett.* **10**, 949–952 (1983).
- Andreae, M. O. & Raedmonck, H. *Science* **221**, 744–747 (1983).
- Andreae, M. O. in *The Biogeochemical Cycling of Sulfur and Nitrogen in the Remote Atmosphere* (eds Galloway, J. N., Charlson, R. J., Andreae, M. O. & Rodhe, H.) 5–25 (Reidel, Dordrecht, 1985).
- Cullis, C. F. & Hirschler, M. M. *Atmos. Envir.* **14**, 1263–1278 (1980).
- Andreae, M. O. *et al. J. geophys. Res.* **90**, 12891–12900 (1985).
- Barnard, W. R., Andreae, M. O., Watkins, W. E., Bingemer, H. & Georgii, H. W. *J. geophys. Res.* **87**, 8787–8793 (1982).
- Logan, J. A., McElroy, M. B., Wofsy, S. C. & Prather, M. J. *Nature* **281**, 185–188 (1979).
- Rodhe, H. & Isaksen, I. *J. geophys. Res.* **85**, 7401–7409 (1980).
- Chatfield, R. B. & Crutzen, P. J. *J. geophys. Res.* **89**, 7111–7132 (1984).
- Gidel, L. T. in *Gas-Liquid Chemistry of Natural Waters* (ed. Newman, L.) pap. no. 6, 1–8 (Brookhaven National Laboratory, Upton, 1984).
- Broecker, W. S. & Peng, T.-H. *Tellus* **26**, 21–35 (1974).
- Perry, R. H. & Chilton, C. H. *Chemical Engineers' Handbook*, 3–234 (McGraw-Hill, New York, 1973).
- Peng, T.-H., Broecker, W. S., Mathieu, G. G. & Li, Y.-H. *J. geophys. Res.* **84**, 2471–2486 (1979).
- Grosjean, D. *Envir. Sci. Technol.* **18**, 460–468 (1984).
- Niki, H., Maker, P. D., Savage, C. M. & Breitenbach, L. P. *Int. J. chem. Kinet.* **15**, 647–654 (1983).
- Baulch, D. L. *et al. J. phys. chem. Ref. Data* **11**, 327–496 (1982).
- Atkinson, R., Pitts, J. N. Jr & Aschmann, S. M. *J. phys. Chem., Ithaca* **88**, 1584–1587 (1984).
- Tuazon, E. C. *et al. J. Phys. Chem., Ithaca* **88**, 3095–3098 (1984).
- Noxon, J. F. *J. geophys. Res.* **88**, 11,017–11,021 (1983).
- Johnston, H. F., Cantrell, C. A. & Calvert, J. G. *J. geophys. Res.* (in the press).
- Winer, A. M., Atkinson, R. & Pitts, J. N. Jr *Science* **224**, 156–158 (1984).
- Maroulis, P. J., Torres, A. L., Goldberg, A. B. & Bandy, A. R. *J. geophys. Res.* **85**, 7345–7349 (1980).
- Huebert, B. J. & Lazrus, A. L. *J. geophys. Res.* **85**, 7337–7344 (1980).

Formation of damage pits by cavitation in a polymer solution

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Cavitation damage is a serious problem in the operation of modern high-speed hydrodynamic machinery. To suppress cavitation inception, polymers have been added to the water¹; however, the effect of polymer addition on cavitation damage^{2–5} has not yet been studied in detail, and there has been no literature on the pitting mechanism in polymer solutions. We show here that the forms and processes of formation of cavitation damage pits in specimens in a polymer solution are totally different from those in water. Oblique and pot-shaped damage pits are formed on a flat, smooth surface of a specimen in a polymer solution. If the specimen has artificial pits such as small conical or cylindrical holes, the parts with small radius of curvature are apt to be damaged, although the smooth surfaces are not. These phenomena are specific to polymer solutions (non-newtonian fluids), and cannot be seen in newtonian fluids such as water.

Vibratory cavitation damage tests (using an aluminium specimen at a resonant frequency of 19.5 kHz and a peak-to-peak amplitude of 38 μm) were performed in water and in a 1,000 p.p.m.w. (parts per million by weight) polyethylene oxide (Polyox WSR-301) aqueous solution. The experimental tester and the procedure have been described in detail⁵. The liquid temperature was 295 K and the viscosity of the polymer solution 2.7×10^{-3} Pa s. By introducing fresh polymer solution into a test vessel at a flow rate of $0.5 \times 10^{-3} \text{ m}^3 \text{ min}^{-1}$, and by discharging

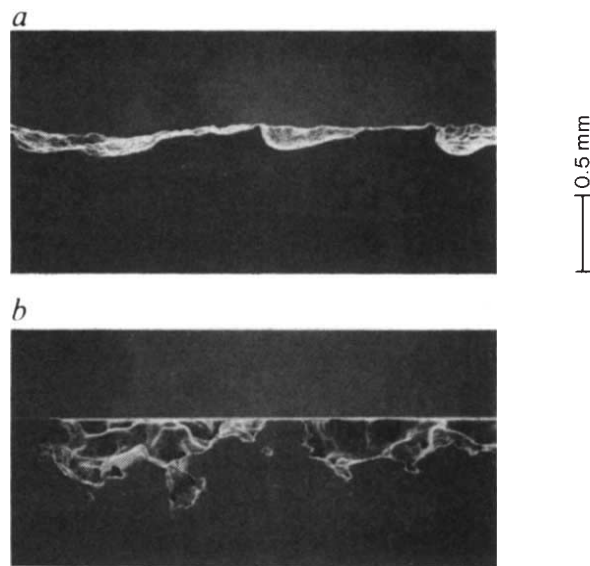


Fig. 1 Cross-sections of aluminium specimens after 60 min exposure to vibratory cavitation in water (a) and in a 1,000 p.p.m.w. Polyox aqueous solution (b). Both photographs are on the same scale. The vibration direction is vertical.

the degraded polymer solution, polymer degradation was significantly suppressed, compared with that of the test solution used by Ashworth and Procter². The test specimen is a cylinder, 15.9 mm in diameter and 12.0 mm high, made of 5056 aluminium with Rockwell hardness $H_{RB} = 50$. Photographs were taken by a scanning electron microscope (SEM).

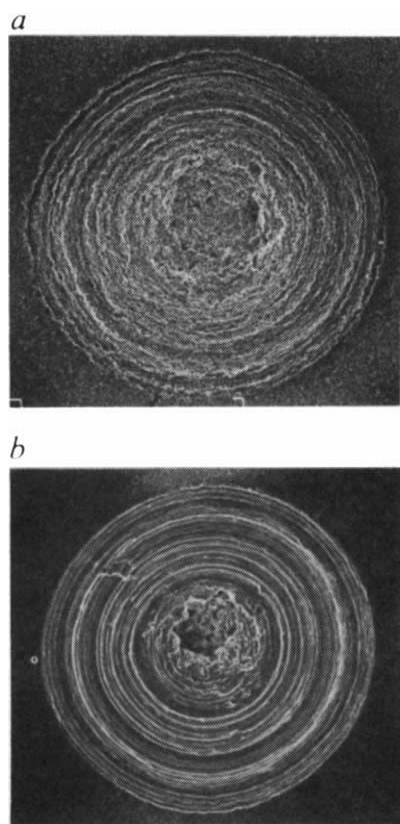


Fig. 2 Conical holes of 1.5 mm base diameter and 0.4 mm depth on a specimen of 15.9 mm diameter, after 5 min exposure to cavitation in water (a) and in a 1,000 p.p.m.w. Polyox aqueous solution (b). Concentric circles are traces made by a drill. In water, these traces were eroded by cavitation damage. In the polymer solution all but the central traces are largely undamaged.

Figure 1 shows cross-sections of specimens after 60 min exposure to cavitation in water and in a 1,000 p.p.m.w. Polyox solution. The side walls of damage pits formed in water are often steeply sloping, but the bottom surfaces are quite flat. In the pits formed in the polymer solution the inside wall of the damaged region is rough and irregular, and a pot-shaped damage pit and damage pits oblique and perpendicular to the vibration direction of the specimen are observed. It seems from the shapes of these pits that the damage pits in the polymer solution are caused by local dynamic pressures, for example from liquid microjets generated by asymmetrical bubble collapses. Moreover, it seems to us that the damage in water depends more strongly on shock waves than does that in the polymer solution.

In order to study the development of damage in pits, damage tests on specimens with 37 small holes were made in water and in a 1,000 p.p.m.w. Polyox solution. Artificial (but representative) damage pits were introduced by drilling conical holes (1.5 mm base diameter and 0.4 mm depth) in the surfaces of the specimens. Figure 2 compares the state of two holes after 5 min exposure to cavitation in water and in the polymer solution. The walls of the conical holes are damaged in water, but not in the polymer solution; the apexes are eroded in both fluids. It thus appears that in the polymer solution only the apex of a pit is strongly eroded, whereas the damage in water progresses in all directions. Since the directions of pitting differ in the two fluids, damage pits formed in polymer solutions are much deeper than those in water, as shown in Fig. 1. The damage rate (0.93 mg min^{-1}) after 3 min exposure to cavitation in the polymer solution is 3.6 times as large as that in water.

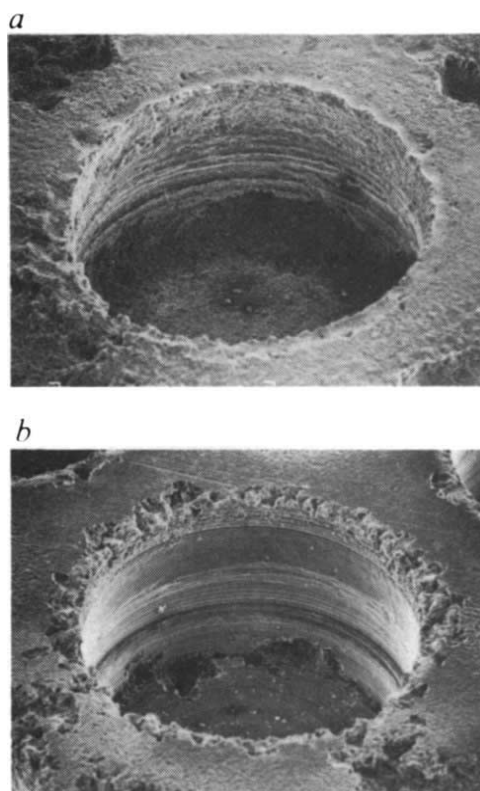


Fig. 3 Cylindrical holes of 1.6 mm diameter and 1.0 mm depth on a specimen of 15.9 mm diameter, after 60 min exposure to cavitation in water (a) and in a 1,000 p.p.m.w. Polyox aqueous solution (b). Photographs are taken oblique to the surface. Circular lines are traces made by an end mill. In water the traces have been eroded, whereas in the polymer solution they are largely undamaged.

The damage patterns in cylindrical holes (1.6 mm diameter and 1.0 mm depth) are shown in Fig. 3. In water, cavitation damage occurs over the entire inside walls of the holes. In the polymer solution, the edges of both the upper and lower surfaces of the cylinders are heavily damaged, but the side walls and bottom surfaces of the holes are undamaged. It may be concluded from the results shown in Figs 2 and 3 that in polymer solutions, regions with small radius of curvature are most susceptible. The Polyox solution used in this work has a strong normal stress effect⁶. In the parts with tight curvature, non-equilibrium normal stresses may occur in the horizontal direction, and bubbles are apt to form. Hence, cavitation damage is more localized in the polymer solution than in water. This behaviour is specific to non-newtonian fluids such as polymer solutions, and cannot be seen in newtonian fluids such as water.

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Received 3 December 1985; accepted 12 February 1986.

1. Ellis, A. T., Waugh, J. G. & Ting, R. Y. *J. bas. Engng* **92**, 459-466 (1970).
2. Ashworth, V. & Procter, R. P. M. *Nature* **258**, 64-66 (1975).
3. Ting, R. Y. *Nature* **262**, 572-573 (1976).
4. Shapoval, I. F. & Shal'nev, K. K. *Soviet Phys. Dokl.* **22**, 635-637 (1977).
5. Shima, A., Tsujino, T., Nanjo, H. & Miura, N. *J. Fluids Engng* **107**, 134-138 (1985).
6. Tsujino, T., Shima, A. & Nanjo, H. *Proc. Instn mech. Engrs* (in the press).

Precious metals in ferromanganese crusts from the south-west Pacific

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Little is known about the abundance, distribution and origin of the precious metals in marine ferromanganese materials¹⁻⁴, although it has long been recognized that elements such as silver and gold are frequently enriched in ferromanganese nodules when compared with average crustal abundance⁵. Relative to crustal abundance, silver is enriched in nodules⁵ by a factor of between 50 and 100, and for gold enrichment factors of between 100 and 1,000 have been found³. We know of no similar data in the literature for silver and gold in ferromanganese crusts, although Halbach *et al.*⁴ have described enrichment of platinum in crusts from the central Pacific region. Elsewhere, precious metals show significant enrichment, possibly to economic grades, in massive sulphide deposits associated with active spreading centres such as the East Pacific Rise and the Red Sea⁶⁻⁸. We now report an unusual occurrence of silver and gold in ferromanganese crusts from a back-arc setting in the south-west Pacific; to our knowledge, the first report of material of this kind.

The ferromanganese crust samples described here were collected from several widely spaced sites on the northeastern and eastern margins of the Indo-Australia Plate (Fig. 1, Table 1). The northernmost deposit (SP10) occurs on the flanks of the South Rennell Trough, situated between the Solomon Islands and New Caledonia, and interpreted by Larue *et al.*⁹ as an Oligocene spreading zone. Sample SP29 is from the north-west margin of the Reinga Basin on the eastern flank of the Norfolk ridge. Sample SP40 is from the eastern flank of the Loyalty Island chain between New Caledonia and Vanuatu. The Loyalty Islands are interpreted by Parrot and Dugas¹⁰ to be the volcanic arc linked to an Eocene to early Oligocene, north-east-dipping subduction zone bordering the Norfolk Ridge. Samples were collected by dredging during the GEORSTOM III NORD (September 1975), ORSTOM EVA I (April–May 1976) and GEORSTOM III SUD (January 1983) cruises, from water depths ranging from 900 to 3,650 m.

Ferromanganese crusts examined here vary considerably in

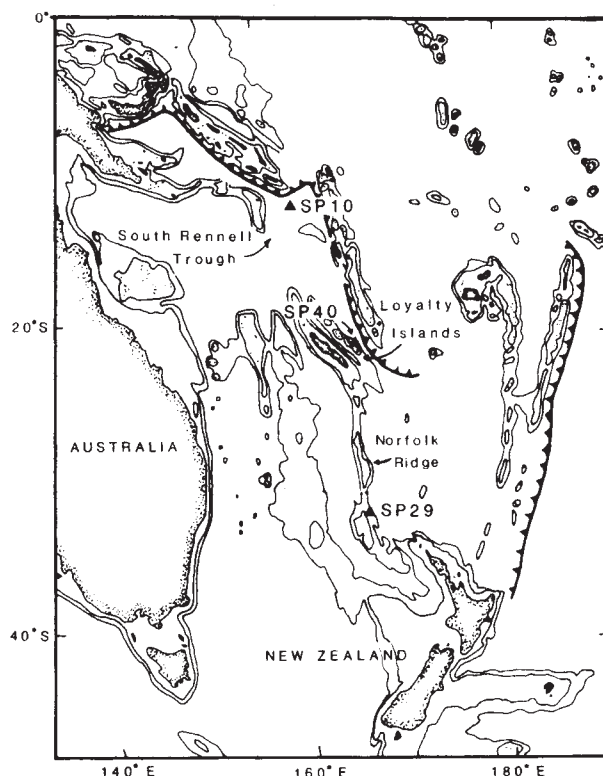


Fig. 1 Map showing location of samples, 1,000- and 2,000-m contours and position of trenches.

surface texture and internal structure. Sample SP10, which is up to 3 cm thick, consists of a slightly gritty and mamillated surface layer with patches of adhering clay, underlain by brownish-black, porous and unlayered ferromanganese material. The substrate for this crust is variably altered basalt, which is separated from the crust by a thin (<1 mm), discontinuous layer of white clay. Crust SP29 consists of a hard, dense and smooth outer layer over black, crudely layered and hard ferromanganese material up to 3 cm thick. The substrate for this crust is bluish-grey chert and chert breccia. Sample SP40 has a rough, gritty surface texture and is composed of brownish-black, coarsely layered ferromanganese material up to 2.5 cm thick. Layering, which occurs on a centimetre scale, is, in part, defined by a higher proportion of light-coloured detrital material in the outermost part of the crust; layers also show a very fine internal lamination, usually <1 mm thick. This crust overlies a mottled, brown to white, gritty clay. The presence of relict pyroxene, magnetite and apatite suggests that this material is probably degraded tholeiitic basalt. Feldspars have generally weathered to clays and recrystallized to phillipsite.

Under high magnification, all crusts studied show features similar to those described for crusts elsewhere^{6,11,12}. The dominant internal structure is a well-defined lamination which frequently forms branching or single digitate columns which may extend through the entire thickness of the crust and are usually perpendicular to the underlying substrate. Cavities between the columns may be filled with clay and entrapped protozoa, usually foraminifera.

Sub-samples of each of the three crusts were analysed by X-ray fluorescence, atomic absorption spectrophotometry, optical microscopy, scanning electron microscopy, X-ray diffractometry, Fourier-transform infrared spectroscopy and Mossbauer spectroscopy. Mineralogically, the crusts are remarkably uniform and consist essentially of X-ray-amorphous ferromanganese material. The only crystalline manganese oxide phase identified in the specimens is ferruginous vernadite, a

Table 1 Bulk chemical composition of crusts from the south-west Pacific* (all analyses in p.p.m. except where stated)

	SP10	SP29	SP40
Latitude	12° 00' S	31° 52' S	20° 47' S
Longitude	162° 43' E	168° 17' E	167° 31' E
Depth (m)	3,100–3,650	900–2,500	1,400–1,700
% Mn	14.8	20.9	14.9
% Fe	16	13.1	16.1
Pb	579	1,573	1,143
Ni	2,329	4,453	2,414
Cu	853	573	1,229
Zn	391	624	539
Co	1,560	6,350	3,720
Mineralogy	Vernadite	Vernadite	Vernadite

* Pb, Ni, Cu and Zn were analysed by X-ray fluorescence using air-dried samples; Mn, Fe and Co were analysed using atomic absorption methods.

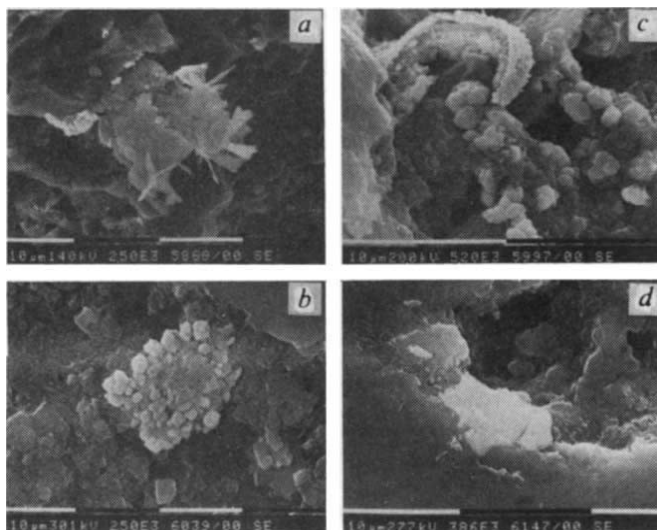


Fig. 2 Scanning electron micrographs of *a*, native silver, centre of field, bordered by radiating crystallites of acanthite; *b*, blocky argentite crystals in clay; *c*, curved particles of cerargyrite (top left) and blocky crystallites of argentite; and *d*, particle of gold, centre of field, in clay. White scale bar, 10 µm in each micrograph.

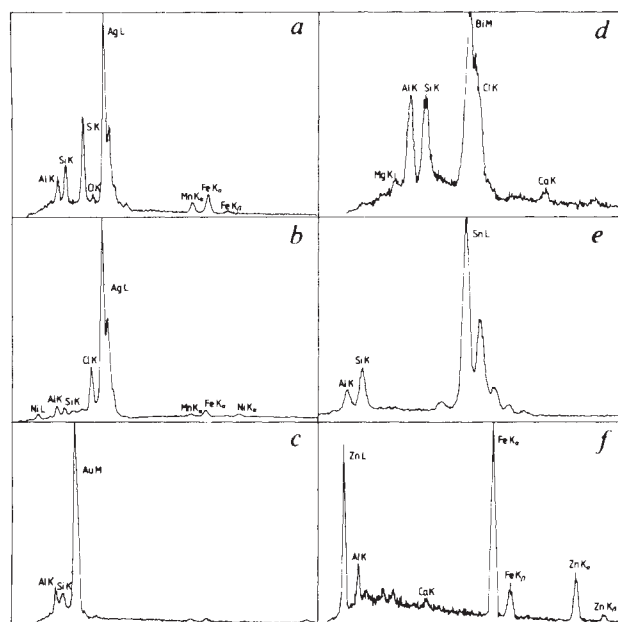


Fig. 3 X-ray energy spectra for *a*, silver sulphide in clay; *b*, silver chloride in clay; *c*, native gold in clay; *d*, bismuth chloride in clay; *e*, cassiterite in clay; and *f*, zinc-iron oxide mineral.

highly hydrated and very-fine-grained iron and manganese oxide^{13,14}.

The results of chemical analysis are given in Table 1. Preliminary analysis for Ag, Au and Pt shows concentration levels below the detection limit for the techniques used (1, 0.05 and 0.25 p.p.m. (parts per 10⁶) respectively). A detailed report on crust mineralogy, chemistry and petrology is in preparation.

Analysis of samples using a scanning electron microscope fitted with an energy-dispersive X-ray system has identified particles of Ag and Au. Silver occurs in a variety of forms, including native silver, silver chloride, a radiating, platy silver sulphide, and an unusual blocky silver sulphide (Fig. 2). Characteristic X-ray energy spectra for the precious metal compounds identified in this study are presented in Fig. 3.

Grains of native silver, the most common variety detected, typically occur in discrete scale-like crystals, irregular in outline and ranging in size from 5 to 50 µm (Fig. 2*a*). The radiating platy silver sulphide has been tentatively identified as acanthite (Ag₂S), on the basis of crystal morphology (Fig. 2*a*). This typically develops as crusts on particles of native silver. A second, blocky silver sulphide (Fig. 2*b*) has been similarly tentatively identified as argentite. This usually occurs as sub-micrometre-sized crystals, often apparently cubic and octahedral, growing from the clay matrix. The silver chloride mineral (Fig. 2*c*) had a very distinctive morphology, consisting of curved particles, suggesting horn silver or cerargyrite (AgCl). This is rarer than the sulphides, and has been observed chiefly on a clay substrate. From 185 silver-containing grains identified by X-ray energy spectroscopy, 143 were native silver, 30 were silver sulphides, and 12 were silver chloride.

Gold, rare compared with silver particles in the samples studied, also occurs in its native form in grains irregular in shape and up to ~10 µm in size (Fig. 2*d*). Also found in crusts and associated with Ag and Au particles are rare grains of SnO₂ (cassiterite), BiCl₃ (mineralogy unknown at present), and an unidentified zinc-iron oxide.

All the above particles are typically found in the argillaceous infilling between the vernadite columns or associated with the decay products of substrate materials. Only rarely do grains appear to be incorporated in the ferruginous vernadite laminae of the columns during their growth.

According to Cronan⁵, major factors affecting rare-element enrichments in nodules and crusts include the adsorptive and crystallochemical characteristics of the ferromanganese oxides themselves, the rate of supply of the elements to the marine environment, their availability at the reaction site, and the nature of the environment of deposition. We have insufficient data to provide a detailed account of the distribution and origin of precious metals in the ferromanganese deposits studied here; however, absorption and substitution are unlikely to have played a major part in Ag and Au enrichment in crusts, as evidenced by the dominance of native metal forms associated with clays and, to a lesser extent, directly incorporated into manganese oxides.

With regard to the source of the elements, submarine volcanism has been seen¹⁵⁻¹⁷ as responsible for the enrichment of some rare elements in nodules from volcanic areas. However, several factors would appear to suggest that submarine hydrothermal processes have not played a role in the growth of the crusts under investigation. First, the vernadite mineralogy of crusts is typical of ferromanganese deposits which have formed by hydrogenous processes, whereby growth is primarily the result of slow, inorganic precipitation of manganese oxides from the near-bottom water^{4,6,18}. In contrast, hydrothermal manganese deposits from active spreading centres and other areas of submarine hydrothermal activity are frequently dominated by birnessite and todorokite^{6,19}. Also, the approximately equal proportion of Mn and Fe and relatively high trace-metal contents in crusts, when compared with material formed by hydrothermal processes, is further evidence, together with their general appearance, that crusts probably have a hydrogenous origin^{6,20}. Nowhere have we observed Ag to be in association with the Fe, Cu and Zn sulphide phases characteristic of hydrothermally derived massive sulphide deposits such as those that are known to occur on the East Pacific Rise⁷.

Previous work on the influence of environmental controls on the distribution and enrichment of Ag in marine sediments has suggested that the most likely removal areas are in nearshore muds or other areas in which oxygen-depleted conditions are maintained. It has been suggested that in these areas Ag is precipitated as silver sulphide after initial supply to the sediment with accumulating organic carbon^{21,22}. A similar mechanism of

enrichment has recently been suggested for Co in ferromanganese crusts forming near areas where the oxygen minimum zone impinges on the flanks of some island chains and seamounts in the Pacific²³. Although data are scarce, we find no convincing evidence for similar environmental controls acting on the crusts under study. Co concentrations are low (Table 1) compared with the crusts mentioned above, and the sediments associated with crusts show no signs of significant oxygen depletion, being bioturbated and light in colour.

The tendency for the Ag and Au particles examined here to occur in clays, either filling voids between vernadite columns or associated with degraded igneous substrate, suggests that at least some of the Ag and Au particles may be of detrital origin. This mode of origin is further supported by the generally irregular shape of the grains and the presence in at least one sample of native silver embedded in slightly altered basalt. However, the delicate, finely crystalline nature of some silver sulphide and chloride particles, together with their apparent intimate relationship to associated clays suggests an authigenic origin. Possible mechanisms for authigenic growth might include diagenetic remobilization and subsequent flocculation of silver colloids from the surrounding clays^{3,24}, or direct precipitation of Ag and Au chloro-complexes, the main species of these metals in the overlying sea water²⁵.

We suggest, therefore, that although the origin of Ag and Au particles in these crusts remains unclear, the major factor influencing their concentration appears to be the presence of a local, metal-enriched igneous source which on degradation has liberated particles of Ag and Au which have, in turn, been entrapped in overlying ferromanganese crusts and associated sediments. Subsequent diagenetic remobilization of some of this Ag has apparently led to the development of authigenic sulphide and chloride phases.

To our knowledge these data represent the first report of particulate silver and gold in intimate association with ferromanganese crusts. Their discovery may have important implications for the offshore resource potential in the south-west Pacific. If further work should locate additional material similar to that described above, and if grade and abundance criteria are met, this material may have economic value.

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- Harriss, R. C. *et al. Geochim. cosmochim. Acta* **32**, 1049-1056 (1968).
- Crockett, J. H. *et al. Geochim. cosmochim. Acta* **37**, 2547-2556 (1973).
- Glasby, G. P. *Mar. Chem.* **1**, 105-125 (1973).
- Halbach, P. *et al. Naturwissenschaften* **71**, 577-579 (1984).
- Cronan, D. S. in *Marine Manganese Deposits* (ed. Glasby, G. P.) 11-44 (Elsevier, Amsterdam, 1977).
- Cronan, D. A. *Underwater Minerals* (Academic, London, 1980).
- Hekinian, R. *et al. Science* **207**, 1433-1444 (1980).
- Bischoff, J. L. & Manheim, F. F. in *Hot Brines and Recent Heavy Metal Deposits in the Red Sea* (eds Degens, E. T. & Ross, D. A.) 535-541 (Springer, New York, 1969).
- Larue, B. M. *et al. Int. Symp. Geodynamics in South-West Pacific* 51-62 (Technip, Paris, 1977).
- Parrott, J. F. & Dugas, F. *Tectonophysics* **66**, 349-372 (1980).
- Raeb, W. & Meylan, M. A. in *Marine Manganese Deposits* (ed. Glasby, G. P.) 109-146 (Elsevier, Amsterdam, 1977).
- Glasby, G. P. & Andrews, J. E. *Pacif. Sci.* **31**, 363-379 (1977).
- Burns, R. G. & Burns, V. M. in *Marine Minerals* (ed. Burns, R. G.) 1-40 (Mineralogical Society of America, Short Course Notes 6, 1979).
- Ostwald, J. *Geol. Mag.* **121**, 483-488 (1984).
- Arrhenius, G. *et al. Science* **144**, 170-173 (1964).
- Harriss, R. C. *Nature* **219**, 54-55 (1968).
- Moorby, S. A. & Cronan, D. S. *Miner. Mag.* **47**, 291-300 (1983).
- Cronan, D. S. *et al. Nature* **298**, 456-458 (1982).
- Corliss, J. B. *et al. Earth planet. Sci. Lett.* **40**, 12-24 (1978).
- Toth, J. R. *Geol. Soc. Am. Bull.* **44**-54 (1980).
- Turekian, K. K. *Geochim. cosmochim. Acta* **32**, 603-612 (1968).
- Martin, J. H. *et al. Nature* **305**, 306-309 (1983).
- Halbach, P. & Puteanus, D. *Earth planet. Sci. Lett.* **68**, 73-87 (1984).
- Krauskopf, K. B. *Econ. Geol.* **46**, 858-870 (1951).
- Riley, J. P. & Chester, R. *Introduction to Marine Chemistry* (Academic, London, 1971).

Contribution of metabolic carbon to mollusc and barnacle shell carbonate

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Carbon in marine calcareous tests is not necessarily derived only from dissolved inorganic carbon (DIC) in ambient sea water; metabolic carbon can also be incorporated into carbonate tests, as shown by experiments using ¹⁴C-labelled food during the incubation of sea urchin embryos and coral^{1,2}. ¹⁴C-rich organic matter (relative to seawater DIC) from terrestrial vegetation as well as marine organic matter (reflecting seawater DIC) is used as food by marine organisms in near-shore environments. This use provides the basis for a natural experiment on the systematics of metabolic carbon incorporation into carbonate tests. Here we have combined ¹⁴C/¹²C and ¹³C/¹²C ratio measurements on both the calcareous and the organic parts of marine organisms and on DIC, plankton and other carbon-bearing materials collected in and around New Haven Harbor (Connecticut, USA) in Long Island Sound, where the various sources of carbon can be identified, and we deduce that a large percentage of the carbon in calcareous tests is metabolic carbon. Thus, it is at best difficult to use the $\delta^{13}\text{C}$ values of ancient biogenic carbonate from molluscs to predict the ancient $\delta^{13}\text{C}$ values of seawater DIC.

Living barnacles and a variety of molluscs were collected during the period 1983-85 at three sites in New Haven Harbor (see Fig. 1). During the same period sea water, sediment, sea-

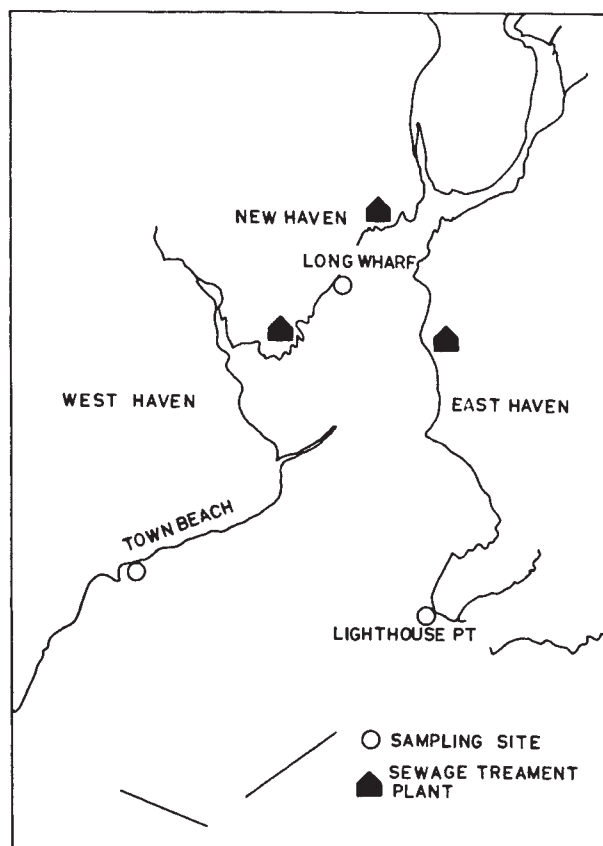


Fig. 1 Sampling sites in New Haven Harbor. The three municipal sewage treatment plants located inside the harbour are indicated.

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Table 1 Analytical results

						Metabolic carbon contribution to shell carbonate	
Sample		$\delta^{13}\text{C}$ (‰)	$\delta^{18}\text{O}$ (‰)	$\delta^{14}\text{C}$ (‰)	Δ (‰)	$M\%$ [$\delta^{13}\text{C}$]	$M\%$ [Δ]
Lighthouse Point, East Haven							
<i>Mytilus edulis</i>	Shell	-2.8	—	+245 ± 14	+190 ± 14	58 ± 5	61 ± 10
	Meat	-20.4	—	+277 ± 7	+265 ± 7		
<i>Crassostrea virginica</i>	Shell	-1.9	-2.9	+201 ± 8	+145 ± 8	43 ± 5	68 ± 17
	Meat	-20.1	—	+191 ± 15	+179 ± 16		
<i>Littorina littorea</i>	Shell	-1.0	—	+216 ± 11	+156 ± 11	73 ± 4	56 ± 12
	Meat	-16.4	—	+245 ± 13	+223 ± 14		
<i>Balanus</i> spp. (barnacles)	Shell	-2.1	—	+148 ± 17	+95 ± 18	56 ± 5	23 ± 22
	Meat	-17.7	—	+187 ± 10	+169 ± 10		
Sea water	DIC	(+0.3)*	—	+131 ± 10	+73 ± 10		
Town Beach, West Haven							
<i>Crassostrea virginica</i>	Shell	-1.8	—	+185 ± 13	+130 ± 13	35 ± 4	36 ± 15
	Meat	-22.3	—	+224 ± 13	+217 ± 14		
Sea water	DIC	(-4.2)*	—	+133 ± 14	+82 ± 14		
Long Wharf, New Haven							
<i>Mytilus edulis</i>	Shell	-2.2	—	+341 ± 16	+279 ± 17	48 ± 5	24 ± 3‡
	Meat	-21.4	—	+867 ± 18‡	+854 ± 19‡		
<i>Nassarius obsoletus</i>	Shell	-1.3	-2.2	+261 ± 10	+201 ± 10	85 ± 3	45 ± 9
	Meat	-15.6	—	+362 ± 11	+336 ± 12		
<i>Crassostrea virginica</i>	Shell	-2.5	-3.7	+255 ± 9	+199 ± 9	45 ± 4	43 ± 9
	Meat	-20.8	—	+355 ± 16	+344 ± 17		
<i>Mya arenaria</i>	Shell	-1.8	-2.0	+299 ± 8	+238 ± 8	63 ± 4	23 ± 3‡
	Meat	-19.0	—	+752 ± 11‡	+730 ± 12‡		
Sediment	Organic-C	-23.5	—	-102 ± 16	-104 ± 6		
Seaweed		-21.2	—	+219 ± 11	+209 ± 11		
Sea water	DIC	(-2.9)*	—	-140 ± 16	+90 ± 17		
Yale Marine Station, Guilford							
Phytoplankton	Organic-C	-17.4	—	+143 ± 12	+125 ± 12		
Sea water	DIC	(0.1)*	—	(+139 to +189)†	(+79 to 127)†		

The Δ values are reported as defined by Stuiver and Polach⁵, following Broecker and Olson⁶ (higher Δ values indicate greater ^{14}C contents and positive Δ values indicate the presence of bomb ^{14}C). $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ are reported relative to the PDB standard. The precision of the carbonate $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ is $\pm 0.1\%$. (1σ) based on replicate analysis. The precision of the meat $\delta^{13}\text{C}$ is $\pm 0.6\%$. (1σ) based on triplicate analysis. The reported Δ value precision is the 1σ quadratic sum of errors associated with the counting statistics of both sample and standards.

* Values in parentheses are the $\delta^{13}\text{C}$ values for CO_2 extracted for ^{14}C analysis. These are used strictly for the calculation of Δ values since they do not represent $\delta^{13}\text{C}$ of seawater DIC.

† A range for seven samples.

‡ These are probably transient values due to ^{14}C -rich tracers introduced into the harbour.

weed and phytoplankton were collected from New Haven Harbor and Long Island Sound near the Yale Peabody Museum Field Station at Guilford, Connecticut.

Mollusc and barnacle meat was separated from shell material immediately after sampling and freeze-dried. CO_2 was extracted from the freeze-dried meat by combustion in a closed system. Approximately 20 g of the shell material was ground in a mortar, roasted in air at 400 °C for 30 min and treated with 2 M HCl in a vacuum system to release CO_2 gas. After the removal of carbonate with 1 M HCl, CO_2 was extracted from the sedimentary organic carbon, seaweed and phytoplankton by combustion in a closed system. CO_2 was extracted from 100 kg of sea water by the addition of sulphuric acid, and the extracted CO_2 was converted to benzene, which was then assayed for ^{14}C activity by liquid scintillation counting.

A small aliquot of the shell material (~10 mg) was roasted at 380 °C for 3 h under vacuum and treated with 100% orthophosphoric acid to release CO_2 . $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of this CO_2 and the CO_2 extracted for ^{14}C analysis were determined on a Nuclide 6-60 ratio mass spectrometer. The Δ , $\delta^{14}\text{C}$, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values are listed in Table 1.

The Δ values (in ‰) of shell carbonate and meat from molluscs collected inside New Haven Harbor (Long Wharf) range from +199 to +279 and +344 to +882, respectively, and are con-

sistently higher than the Δ values of shell carbonate and meat from molluscs collected at the mouth of the harbour (Lighthouse Point and Town Beach), which range from +130 to +190 and +179 to +265, respectively (see Table 1). The meat of *Mytilus edulis* and *Mya arenaria* collected at Long Wharf was extremely rich in ^{14}C , with Δ values of +882 to +730, respectively. The ^{14}C -rich meat indicates a source of ^{14}C other than the atmospherically derived carbon found in plants and the sewage sludge. The Δ values of DIC from seawater collected throughout New Haven Harbor are quite constant (+82 ± 10) and much lower than the Δ values of either mollusc shell carbonate.

The contrast between the low and constant Δ values of New Haven Harbor seawater DIC and the variable and high Δ values of mollusc shells collected both at a single site (Long Wharf) and at several sites within New Haven Harbor, indicates that the carbon in the shell carbonate is derived from a source other than seawater DIC. Pore water DIC and metabolic carbon are the only other sources of carbon available for incorporation into shell carbonate. As the clams analysed are primarily epifaunal and as the Δ values of the infaunal and epifaunal clams at any one site are not significantly different, the pore water option is eliminated.

Sewage sludge egressing from the three municipal sewage sludge treatment plants located in the inner harbour (see Fig. 1)

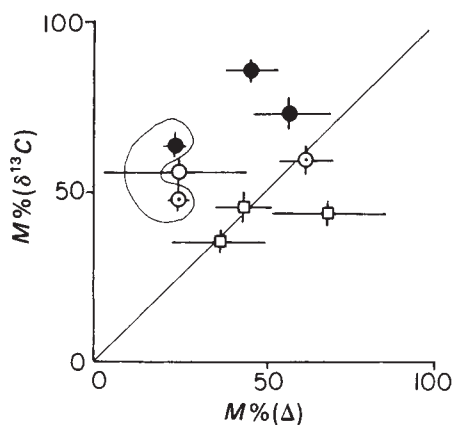


Fig. 2 Relationship between $M\%[\Delta]$ and $M\%[\delta^{13}\text{C}]$. The circled data are from *Mytilus edulis* and *Mya arenaria* collected at Long Wharf. The Δ values for these samples are suspected to be in a transient state. A temperature of 20 °C during carbonate deposition was assumed for the calculation, which required an ϵ_{s-b} of +1.85‰ (ref. 7) and an ϵ_{s-a} of +11.4‰ (ref. 8) for calcitic shells (*C. virginica* and *Ballanus* spp.). For aragonitic shells (*L. littorea*, *M. arenaria* and *N. obsoletus*), 1.8‰ greater fractionation factor than that for calcitic shells was used⁹. *M. edulis* has a shell made of both calcite and aragonite, a ratio to calcite and aragonite of 1:1 was assumed, resulting in a 0.9‰ greater fractionation factor than that used for calcitic animals. The $\delta^{13}\text{C}$ value of HCO_3^- in sea water assumed in the calculation is estimated to be $+0.5 \pm 0.5\%$ based on a salinity of 20‰ to 25‰ observed in New Haven Harbor and the $\delta^{13}\text{C}_{\text{DIC}}$ -salinity relationship observed in Chesapeake Bay by Spiker¹⁰. □, Calcitic; ●, aragonitic; ○, calcitic/aragonitic; ○, barnacle (calcitic).

is a likely source of the metabolic carbon, as it has a high Δ value reflecting the contemporary atmospheric Δ value. A Δ value of +430 has been reported for sewage sludge egressing from the West Haven sewage treatment plant³, and this would also be the value of contemporaneous terrestrial vegetation supplied to the harbour. Additional sources of metabolic carbon include plankton and seaweed. The Δ value of phytoplankton does not deviate from those of DIC (see Table 1), suggesting that phytoplankton is not a source of ^{14}C -rich carbon. The Δ value of seaweed collected in the intertidal zone at Long Wharf is +209, indicating both the intertidal seaweed uses ^{14}C -rich atmospheric CO_2 during photosynthesis and that the consumption of this seaweed by molluscs could be reflected in the ^{14}C content of their shell material.

Throughout the harbour, the ^{14}C content of mollusc and barnacle shell carbonate is lower than that of their meat and higher than that of DIC, indicating a mixture of carbon from metabolic and DIC sources. Assuming that the distribution of ^{14}C between meat, shell carbonate and DIC is in a steady state, and that the meat Δ values represent the Δ values of metabolic carbon, the per cent ($M\%[\Delta]$) of carbon in shell carbonate derived by the metabolism of food can be calculated using a simple two-endmember mass balance model:

$$M\%[\Delta] = \frac{\Delta_{\text{shell}} - \Delta_{\text{DIC}}}{\Delta_{\text{meat}} - \Delta_{\text{DIC}}} \times 100 \quad (1)$$

Results using this model indicate that 23–68% of the total carbon in the shell carbonate is derived from metabolic sources (Table 1). No species-dependency of the $M\%[\Delta]$ in the shell carbonate is apparent. The high Δ values and low model-derived $M\%[\Delta]$ in shell carbonate of the *M. edulis* and *M. arenaria* collected at Long Wharf, in comparison with the percentage of metabolic carbon calculated for other molluscs, indicate that the ^{14}C content of mollusc meat and shell carbonate may be in a transient state, due to variation in the ^{14}C content of the mollusc's food. Therefore, the assumption of steady state used in the mass

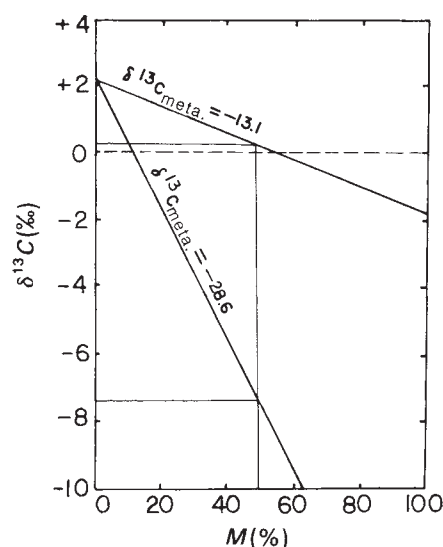


Fig. 3 Effect of incorporation of metabolic carbon into calcitic shell carbonate on the $\delta^{13}\text{C}$ value of shell carbonate. The calculation assumes that the shell was deposited in sea water with a DIC $\delta^{13}\text{C}$ value of 0‰ at 20 °C. The upper and lower lines correspond to incorporation of $\delta^{13}\text{C}_{\text{meta}}$ values of -13.1‰ and -28.6‰, respectively.

balance model is not strictly valid when applied to molluscs eating food that has variable ^{14}C contents, such as sewage, which includes ^{14}C -labelled materials from medical and research facilities.

Assuming isotopic and chemical equilibria among DIC species used during shell carbonate deposition, the percentage ($M\%[\delta^{13}\text{C}]$) of metabolically derived carbon in shell carbonate can also be assessed using a mass balance model which relates the $\delta^{13}\text{C}$ values of shell carbonate to the $\delta^{13}\text{C}$ values of seawater DIC and the metabolic carbon reservoirs:

$$\delta^{13}\text{C}_{\text{shell}} = (\delta^{13}\text{C}_{\text{b,sw}} + \epsilon_{s-b}) \left(1 - \frac{M\%[\delta^{13}\text{C}]}{100} \right) + (\delta^{13}\text{C}_{\text{meta}} + \epsilon_{s-a}) \frac{M\%[\delta^{13}\text{C}]}{100} \quad (2)$$

where $\delta^{13}\text{C}_{\text{b,sw}}$ and $\delta^{13}\text{C}_{\text{meta}}$ represent the $\delta^{13}\text{C}$ values of bicarbonate in the ambient seawater and of the metabolically produced CO_2 , respectively. Because $\delta^{13}\text{C}_{\text{meta}}$ cannot be measured directly, $\delta^{13}\text{C}$ values of meat were used for the calculation. ϵ_{s-b} is the per mil fractionation factor between HCO_3^- and CaCO_3 , and ϵ_{s-a} is the factor between aqueous CO_2 and CaCO_3 .

The $M\%[\delta^{13}\text{C}]$ values obtained from the model range from 35 to 85%, with a mean of 56%. These values are compatible with the $M\%[\Delta]$ value obtained from the ^{14}C data, which range from 23 to 68%, with a mean of 42%. The $M\%[\delta^{13}\text{C}]$ values are plotted against the $M\%[\Delta]$ values for each organism in Fig. 2. The $\delta^{13}\text{C}$ and Δ data produce results that are distributed similarly and centre on about 50%. However, the data do not agree in detail because they do not fall on the predicted slope of 45°. This result is not unexpected because: (1) Δ values of the meat of *M. edulis* and *M. arenaria* collected at Long Wharf are likely to represent transient values; and (2) the aragonitic fractionation factor for ^{13}C is not well constrained. All data collected from calcitic molluscs within the analytical precision of the measurements fall on the line of 45° slope in Fig. 2; however, all data from aragonitic animals fall clearly above the line of 45° slope. This indicates either that the aragonitic fractionation factor which has been reported is too large, or that aragonite was not precipitated in isotopic equilibrium. Nevertheless, even though there are uncertainties, it is clear that both $^{14}\text{C}/^{12}\text{C}$ and

$^{13}\text{C}/^{12}\text{C}$ data indicate that ~50% of the carbon in mollusc and barnacle shell carbonate are derived from metabolic carbon.

Peterson *et al.*⁴ have shown that the $\delta^{13}\text{C}$ value of meat from ribbed mussels (*Geukensia demissa*) in salt marshes reflects the complete range of $\delta^{13}\text{C}$ in the local food sources. The values vary from -13.1 to -21.3‰, reflecting a mixture of *Spartina* (C_4) marsh grass and plankton. These workers further point out that in an area with high riverine input, upland C_3 plants, with a $\delta^{13}\text{C}$ value of -28.6‰ (the value used by Peterson *et al.*⁴) are also likely to be consumed. Therefore, the expected $\delta^{13}\text{C}$ variation due to incorporation of metabolic carbon can be expected to be large.

The expected effect of metabolic carbon incorporation into shell carbonate on the shell carbonate $\delta^{13}\text{C}$ value can be demonstrated by using equation (2). The expected $\delta^{13}\text{C}$ value of shell carbonate for different values of incorporated metabolic carbon are shown in Fig. 3. This shows that it is possible for the shell carbonate to have both lighter and heavier $\delta^{13}\text{C}$ values than that of the ambient seawater DIC. For instance, if no metabolic carbon is incorporated into the shell, the $\delta^{13}\text{C}$ value of the shell should be ~2.0‰ heavier than the $\delta^{13}\text{C}$ value of DIC as the result of equilibrium partitioning of the isotopes. However, if 50% of the carbon in the shell is metabolic in origin, the $\delta^{13}\text{C}$ value can vary between -0.3 and 7.5‰ lighter than the ambient DIC, depending on the isotopic composition of the local food source.

The above discussion indicates that, for molluscs, a 15.5% variation in the $\delta^{13}\text{C}$ value of food, which can easily be expected

due to the change of eating habits in time and space, translates into a 7.8‰ variation in the shell carbonate $\delta^{13}\text{C}$ value. This result suggests that it is difficult at best to use the $\delta^{13}\text{C}$ values of ancient biological carbonate from molluscs to predict the ancient $\delta^{13}\text{C}$ values of seawater DIC. If the carbon isotopic systematics of other organisms, for example, foraminifera and pteropods, are similarly affected, the variability in their carbon isotopic signatures cannot simply be taken to indicate the carbon isotopic composition of seawater DIC in which they grew. However, if the food source does not vary in isotopic composition and if these animals always incorporate roughly the same percentage of metabolic carbon into their shells, the relative change in the $\delta^{13}\text{C}$ value of DIC through time and space will be preserved.

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1. Pearse, V. B. *Nature* **228**, 383 (1970).
2. Sikes, C. S., Okazaki, K. & Fink, R. D. *Comp. Biochem. Physiol.* **70A**, 285-291 (1981).
3. Benoit, G. J., Turekian, K. K. & Benninger, L. K. *Estuar. coast. Shelf Sci.* **9**, 171-180 (1979).
4. Peterson, B. J., Howarth, R. W. & Garritt, R. H. *Science* **227**, 1361-1363 (1985).
5. Stuiver, M. & Polach, H. A. *Radiocarbon* **19**, 355-363 (1977).
6. Broecker, W. C. & Olson, E. A. *Radiocarbon* **3**, 176-204 (1961).
7. Emrich, K., Ehalt, D. H. & Vogel, J. G. *Earth planet. Sci. Lett.* **8**, 363-371 (1970).
8. Mook, W. G., Bommerson, J. C. & Staverman, W. H. *Earth planet. Sci. Lett.* **22**, 169-176 (1974).
9. Robinson, M. & Clayton, R. N. *Geochim. cosmochim. Acta* **33**, 997-1002 (1969).
10. Spiker, E. C. *Radiocarbon* **22**, 647-654 (1980).

Long-range colour-generating interactions across the retina

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The existence of colour-generating interactions across the corpus callosum has recently been suggested from observations with a 'split-brain' patient¹, thus indicating long-range colour computations at the cortical level. Observations on induced colours described here suggest long-range colour computations at the retinal level. If a white surface surrounded by a particular colour is fixated for some time, the resulting after-image has two colours: the surround appears in complementary colour, whereas the white centre takes on the colour of the surround. The question of whether such colour induction is located in the retina or more centrally was tested in a brain-injured patient with hemianopia. It could be demonstrated that areas of the visual field that are no longer represented in the geniculostriatal pathway still contribute to colour induction, suggesting that colour induction is a retinal phenomenon.

If one fixates a coloured surface for several seconds, one can see an after-image of complementary colour if one then looks at a homogeneous white surface (see Fig. 1). It has been assumed that complementary after-images are a consequence of fatigue within a receptor population that is sensitive to a certain band of the visible spectrum. Presumably, fatigue leads to an excess of activity within a receptor population that is sensitive to another band of the spectrum. This excess of activity results in an after-image of complementary colour. Adjacent surfaces, however, are also affected in their appearance in the after-image condition, as von Helmholtz has pointed out². Induction leads to a homogeneously coloured pattern with no indication of more (or less) saturation at the border towards the region that appears in complementary colour.

The spatial limits of colour induction are difficult to determine. A central white area with a radius of 10° visual angle (or even

more) is still filled by the colour of the surround; saturation, however, decreases with increasing radius of the inducing frame. What colour will be induced if the surround is composed of different colours? If the surround is made up of red, green, yellow and blue, the induced colour in the after-image condition will be grey, suggesting a homogeneous mixture of the various colours of the surround. It is interesting that in this situation, again the induced surface appears homogeneously throughout, with no indication of a colour emphasis towards the regions of the inducing colours.

It is proposed that long-range colour induction can be explained on the basis of regional inhibition. Fatigue within a receptor population that is tuned to a certain band of the spectrum not only leads to an imbalance of neuronal activity within the stimulated area itself (resulting in a complementary after-image), but also influences colour analysis in adjacent areas and possibly in the entire receptive surface. Whenever there is fatigue within a specific receptor population, regional inhibition within this 'colour channel' will be reduced. Reduction of regional inhibition will lead to an increase of sensitivity within this colour channel for adjacent areas of the receptive surface. This sensitization in the adjacent areas is responsible for the induced colour, that is, the spatial carry-over of a neighbouring colour. As colour induction can be observed across large distances within the visual field, a rather extensive inhibitory network must be assumed.

It has been demonstrated that after-images are retinal in origin³. A simple demonstration indicating the retinal basis of colour induction was obtained with the help of a patient who had suffered an injury within the geniculostriatal projection system which had resulted in an incomplete homonymous hemianopia on the right side of the visual field (Fig. 2). The shaded region on the right side of Fig. 2 indicates blindness. In the perifoveal region, blindness starts at the vertical meridian and there is no indication of foveal sparing. On the basis of computer tomography, and because this patient does not show residual vision⁴⁻⁶, it can be safely assumed that the shaded region has no cortical representation. With this patient, experiments on colour induction were performed in which parts of the patterns were presented within the blind region of the visual field.

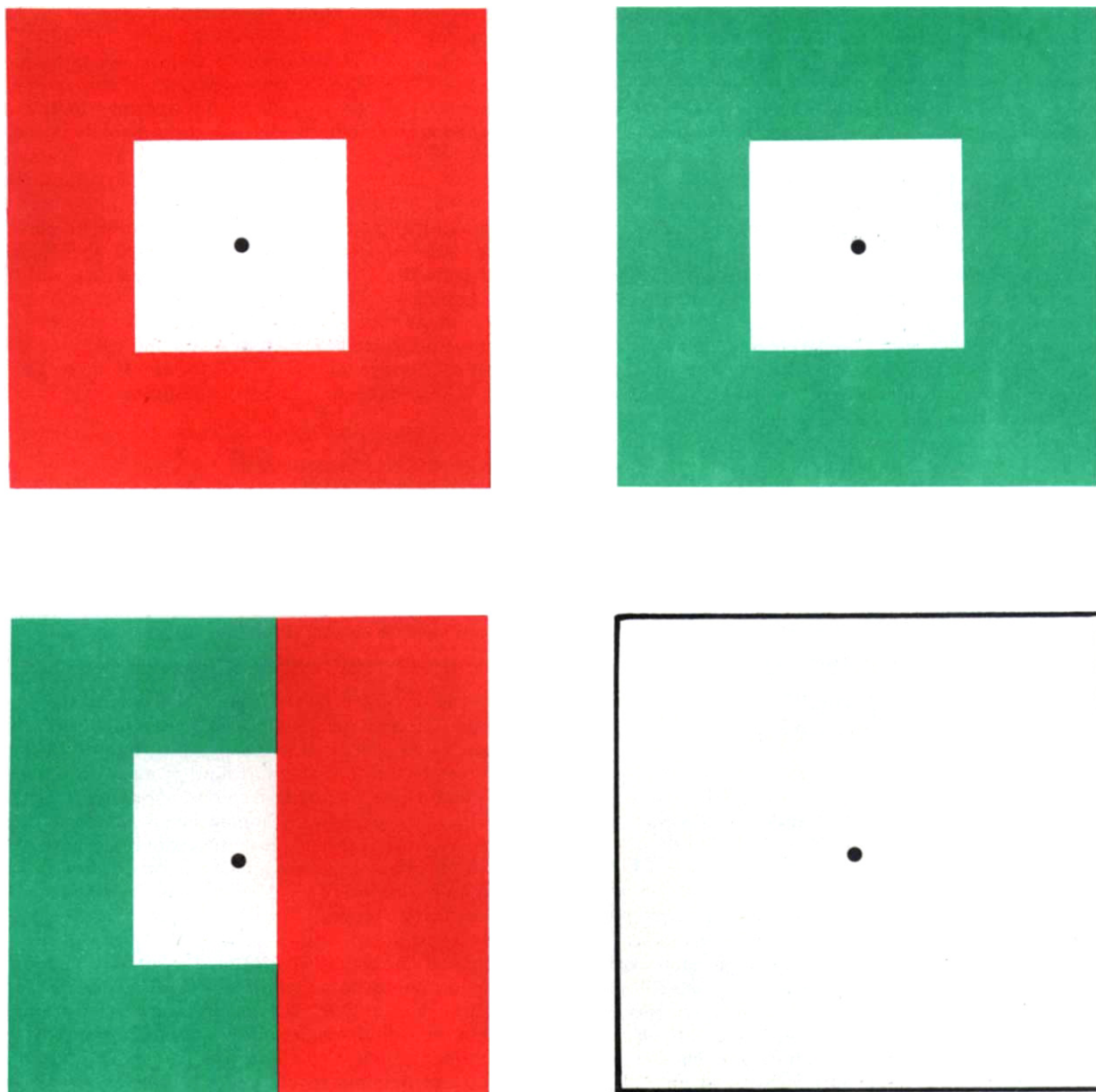


Fig. 1 Colour patterns used to induce complementary after-images and induced colours. Induced colours may be visualized as follows. Fixate on the dot at the centre of the red or green frame for several seconds, then fixate the dot in the blank square (lower right). The after-image that appears will have the surrounding frame in the complementary colour, whereas the white centre takes on the colour of the original surround.

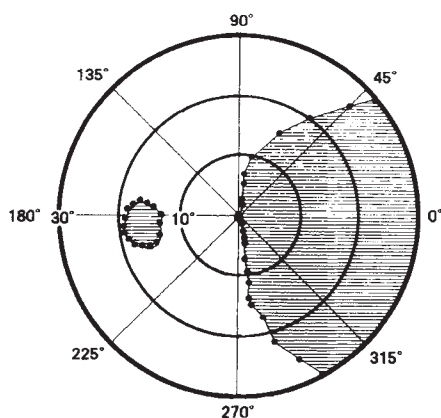


Fig. 2 Central region of the visual field (OS; left eye) of a patient who has suffered a left hemisphere injury resulting in an incomplete hemianopia on the right side (shaded region). The visual field was measured using a 10-arc min stimulus of 32 cd m^{-2} luminance and a background luminance of 3.2 cd m^{-2} . Note that in the perifoveal region the area of blindness begins at the vertical meridian and that there is no foveal sparing. The shaded region to the left of the vertical meridian indicates the position of the blind spot.

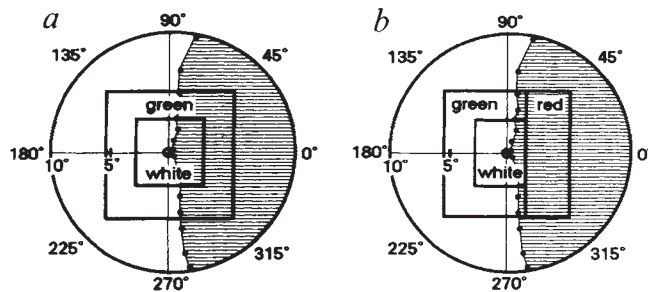


Fig. 3 Stimulus arrangement for colour induction in the patient with a hemianopia shown in Fig. 2. Only the central region of the visual field ($r=10^\circ$) is shown. *a*, A white square with a green surround was positioned in such a way that a large part of the pattern was located in the blind region of the visual field. *b*, Most of the pattern that was represented in the blind region was changed to red. Both patterns were used in this spatial configuration for colour induction. Although the patient could not see any difference between the two patterns when looking at the fixation point, he reported for their centres a marked difference of induced colour in the after-image condition.

Stimuli used in these experiments are shown in Fig. 1 and the stimulus arrangement is demonstrated in Fig. 3. Parts of the central white area and surround cannot be seen by the patient when he looks at the fixation point, because they are projected into the blind field. If the patient fixates the centre of the pattern with a green surround (Figs 1*b*, 3*a*) for several seconds, he will report that the central region of the pattern that is visible to him has turned green in the after-image condition. In the next step (Fig. 3*b*), the part of the pattern that lies in his blind region is changed to red. (Fig. 1*c*). For the patient, the two patterns look identical but the induced colours in the central regions are different. Like any normal observer, the patient reports that the induced central region in Fig. 3*b* is considerably darker and looks 'greyish' compared with Fig. 3*a*. Thus, a part of the visual field that presumably has no representation in striate and extrastriate cortex can alter the induced colour. On the basis of this observation, it must be concluded that colour induction is mediated by the retina.

One important aspect of the retinex theory of colour vision is the idea that long-range interactions across the visual field must be assumed⁷. Although it has been found that the cortex is necessary for long-range colour computations¹, the observations on colour induction reported here point to the retina as being an important part of the neuronal machinery for colour computation. If the phenomenon of colour induction is related to the long-range interactions required in the retinex theory, one might speculate that the so-called designators for the long, middle and short wavebands are already determined in the retina whereas the construction of colours on the basis of the designators is a cortical phenomenon. This decomposition of the retinex algorithm into two structural components would allow a common explanation for the long-range colour induction described here and the long-range colour-generating interactions at the cortical level that have been suggested previously¹.

I thank the patient F.S. for his willingness to participate in these experiments and Gabi Weiler for her assistance. This work was supported by DFG (PO 121/10).

Colour-contingent after-effects are really wavelength-contingent

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McCollough¹ reported that following adaptation to (say) a red and black pattern of vertical stripes, alternating every few seconds with a green and black pattern of horizontal stripes, an orientation-contingent colour after-effect is observed when black and white gratings are viewed. Vertical gratings are tinged with green and horizontal gratings with pink. We have exploited colour constancy, the tendency for objects to appear constant in hue despite large changes in the spectral composition of the illuminant, to examine whether the colours observed on the McCollough effect test gratings are determined by the wavelength composition of the adaptation patterns or by their perceived colour. The key to this approach can be illustrated by Edwin Land's elegant demonstrations of colour constancy using 'Mondrian' displays². By embedding the adapting grating that is used to induce the McCollough effect within a Mondrian we show that the effect depends upon the wavelength of light coming from the grating, rather than the perceived colour.

In Mondrian displays colour constancy is demonstrated by illuminating a random collection of coloured rectangles of matt papers, a 'Mondrian', with three bands of wavelengths whose relative energies are variable. A piece of paper which we would describe as being red in white light will continue to appear red when embedded within the Mondrian even when the illuminant is predominantly green and hence little long-wavelength light is being reflected from the paper. The logic of our experiment is now easily explained. The red and black vertical adaptation grating of the McCollough effect was embedded in a Mondrian background and the whole illuminated with predominantly middle-wavelength light so that, while the embedded grating

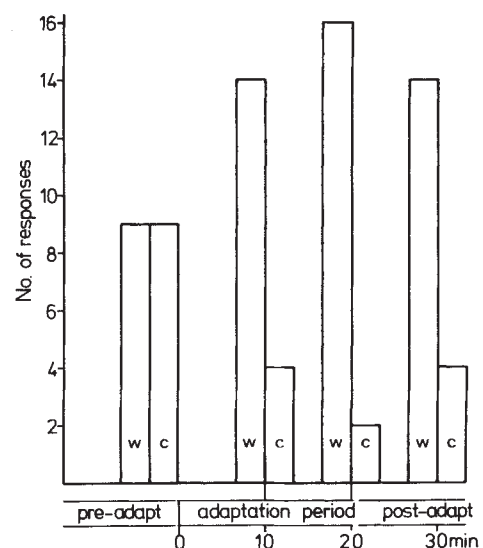


Fig. 1 Subject responses to achromatic vertical and horizontal test gratings before, during and after adaptation. Bars marked 'W' show number of subjects (out of a total of 18) giving response appropriate to contingency between adaptation wavelength and orientation, bars marked 'C' those appropriate to contingency between perceived colour and orientation. The pre-adapt histograms show that there was no bias among the subjects to see either orientation of grating tinged with pink or green prior to adaptation.

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- Land, E. H., Hubel, D. H., Livingstone, M. S. Perry, S. H. & Burns, M. M. *Nature* **303**, 616-618 (1983).
- von Helmholtz, H. *Handbuch der Physiologischen Optik* 2nd edn (Leopold Voss, Hamburg, 1896).
- Craik, K. J. W. *Nature* **145**, 512 (1940).
- Pöppel, E., Held, R. & Frost, D. *Nature* **243**, 295-296 (1973).
- Weiskrantz, L., Warrington, E. K., Sanders, M. D. & Marshall, J. *Brain* **97**, 709-728 (1974).
- Pöppel, E. *Neurosci. Res. Prog. Bull.* **15**, 335-343 (1977).
- Land, E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5163-5169 (1983).

still looks red it is actually reflecting more middle- than long-wavelength light. This display was alternated with the same Mondrian with an embedded green and black horizontal grating, the whole illuminated with a predominantly red light. Thus the vertical grating looks red but reflects predominantly middle-wavelength light while the horizontal grating looks green but reflects predominantly long-wavelength light. This adaptation sequence might establish a contingency between long wavelengths and horizontal gratings and between middle wavelengths and vertical gratings. On the other hand, it might establish a contingency between 'experiential red' and verticals and between 'experiential green' and horizontals. These two possibilities predict opposite after-effects when viewing a black and white test pattern comprising vertical and horizontal gratings.

Subjects sat 114 cm from the display, at which distance the Mondrian subtended 20 degrees of visual angle both vertically and horizontally. The adaptation and test patterns were 2-cycle-per-degree square-wave gratings occupying the central 4×3 degrees. The display was illuminated by two projectors: one illuminated the whole display throughout and was filtered by a neutral-density filter so that a white square on the Mondrian had a luminance of approximately 8 candela m^{-2} . The second projector was filtered alternately by Ilford narrow-cut tricolour filters 205 (red) and 408 (green) during the adaptation phase of the experiment and was unfiltered during the test phase. Both the Mondrian and the gratings were constructed from matt papers. No measurement of the spectral composition of the light reflected from the adaptation patterns was made. However, when the bars of the adaptation patterns were viewed through pin-hole apertures, colour constancy broke down and the gratings illuminated by long-wavelength light appeared red, those by middle-wavelength light, green.

The experiment involved 18 subjects, 9 adapted to the vertical grating in 'red' light and 9 in 'green' light. Prior to adaptation a forced-choice judgement was demanded of each subject as to the perceived colour of the vertical part of the test grating illuminated in white light. All subjects protested that there was no colour to be seen and when given a forced-choice between red and green 9 subjects chose red and 9 green.

Subjects were shown the test gratings again in white light after 10 minutes of adaptation, after a second period of 10 minutes of adaptation, and for a final time 10 minutes after the adaptation period. On each test the subject was asked to make a forced choice to decide which orientation in the test pattern, vertical or horizontal, appeared pink and which green. Therefore the experimental sequence was:

Pretest/10 min adapt/Test 1/10 min adapt/Test 2/10 min rest/Test 3

The results (Fig. 1) show that after 10 minutes of adaptation, 14 out of 18 subjects reported that vertical and horizontal test gratings appeared tinged with the same colour as had been paired with that orientation in the adaptation stimuli. That is, following adaptation to vertical gratings which appeared red during adaptation, vertical test gratings appeared tinged with pink. After a further 10 minutes this had risen to 16 out of 18, and after a 10 minute rest 14/18 still reported the effect.

This result shows that the contingency which has been established is between the orientation of the gratings and the wavelength of the reflected light and not between the orientation and perceived colour of the adaptation pattern. Could this tell us anything about the physiological locus of the McCollough effect? Zeki³ has reported that whereas the response of cells in V1 depends on the wavelength of light falling on their receptive fields, some cells in V4 appear to exhibit colour constancy, responding to the colour of objects rather than to reflected wavelengths. The present result appears to implicate cells of the sort encountered in V1, and not the colour constancy cells of V4. However, an intriguing alternative interpretation might suggest the very opposite. When a red vertical grating is illuminated

in a green light the observers behave as if they learn to compensate for the excess of green light when they view the vertical test grating, rendering it pink. Couched in such terms, the McCollough effect could be seen as a form of orientation-contingent colour constancy, and hence might be regarded as arising in cells of the type Zeki reports in V4.

These data were first presented to the 7th European Conference on Visual Perception, Cambridge, 1984. We thank Dr John Mollon for many suggestions, particularly the interpretation of the effect as a form of orientation-contingent colour constancy; and David Travis for valuable comments.

Received 18 September 1985; accepted 3 February 1986.

1. McCollough, C. *Science* **149**, 1115-1116 (1965).
2. Land, E. H. *Scient. Am.* **237**, 108-129 (1977).
3. Zeki, S. *Nature* **284**, 412-418 (1980).

Traction proteins in the extracellular matrix of *Dictyostelium discoideum* slugs

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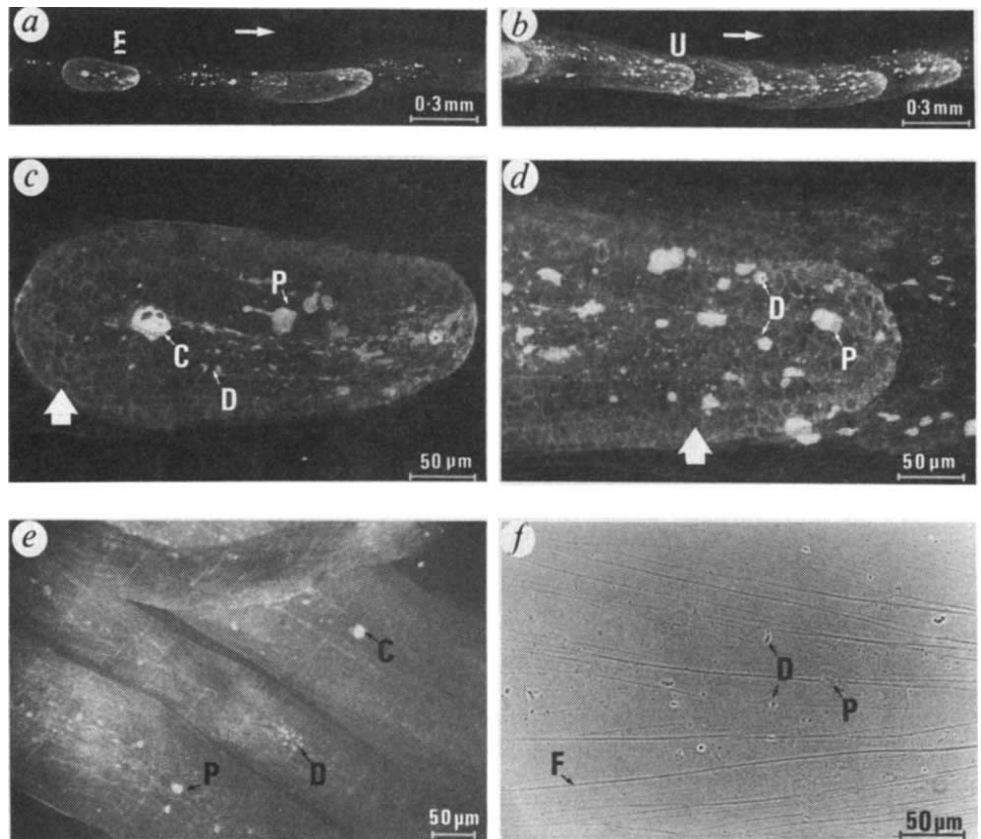
In developing embryos, cells interact with their environment via an extracellular matrix (ECM) which may also provide positional information and directional guidance^{1,2}. Such interactions are difficult to study *in vivo* because the ECM is inaccessible. These difficulties can be avoided with *Dictyostelium discoideum*. The slug of this simple multicellular organism resembles a tiny sausage whose sheath is a continuously produced ECM of cellulose and protein³⁻⁶ through which the cells move. Behind the advancing slug, the empty sheath collapses to leave an easily accessed trail of relatively pure ECM. Here we report studies with a monoclonal antibody which reveals striking patterns in the trail of the migrating slug. These patterns contain cell prints which indicate the former positions of slug cells within the sheath. This leads us to re-evaluate how *D. discoideum* cells move with respect to their ECM^{7,8} and to propose the existence of a class of 'traction proteins'.

MUD50 is a monoclonal antibody which recognizes several cell and trail (ECM) proteins in *D. discoideum*^{9,10}. When trails are treated with MUD50 (see Fig. 1 legend), patterns are observed at irregular intervals on the lower surface of the lower layer of each trail (Fig. 1a, b). These patterns are of two types, elliptical and U-shaped. Generally, elliptical patterns are widely spaced and are associated with small slugs, although occasionally the trails of large slugs show ellipses (see Fig. 2). U-shaped patterns are more closely spaced and are associated with large slugs. The length of each type of pattern is considerably less than that of the corresponding slug.

At high magnification, reticular 'cell prints' are clearly evident within each pattern (Fig. 1c, d). These prints are seen only when the lower surface of a trail is exposed to MUD50. When the upper surface is exposed to the antibody, no prints are seen because the lower surface of the lower layer of trail is adherent to the glass of the microscope slide and thus the cell print antigens cannot react with MUD50 (Fig. 1e). Within the trails, cells and cell debris fluoresce brightly when the lower surface is exposed to MUD50 and less brightly when the upper surface is so treated (Fig. 1d, e). It is possible that the upper layer of

Fig. 1 Patterns in slime trails. *a, b*, The lower surfaces of two trails treated with MUD50. Small arrows show the directions of travel. E, elliptical pattern; U, U-shaped pattern. *c*, Detail of elliptical pattern in *a*. Cell prints (large arrow), cells (C), cellular debris (D) and patches of proteins (P) fluoresce brightly following MUD50 treatment. *d*, Detail of U-shaped pattern in *b*. *e*, The upper surfaces of several trails treated with MUD50. Cells (C), cellular debris (D) and patches of protein (P) fluoresce brightly as in *c* and *d* but cell prints are absent. *f*, Phase-contrast micrograph of *d*. Note the folds (F) in the collapsed sheath. Cell prints can be reproduced here only if the contrast of the micrograph is greatly enhanced.

Methods. Preparation of slugs: Vegetative amoebae were collected into Bonner's salt solution (BSS) from a nutrient agar plate bearing *Dictyostelium discoideum* (strain WS380B) which had been grown at $21 \pm 1^\circ\text{C}$ on *Klebsiella aerogenes*¹¹. Amoebae were separated from the bacteria by washing and centrifugation, then 25- μl aliquots of BSS each containing 10^6 amoebae were put down on water agar plates so that each aliquot formed a circular patch. After drying, plates were covered and sealed in black boxes. Cells aggregated to form slugs which migrated across the agar towards light entering a small hole drilled in the side of each black box¹². Preparation of slides: Slime trails adhere relatively well to hydrophobic slides. Cleaned or hydrophilic slides can be made adherent by bubbling in a chrome alum/gelatin solution¹³. Exposing the lower surfaces of trails: Three days after aggregation, when slugs had formed and migrated across a water agar plate, a hydrophobic microscope slide was pressed down onto the surface of the agar plate. The upper surfaces of the slime trails adhered to the glass. When the slide was lifted, the trails were removed from the agar with their lower surfaces exposed. Exposing the upper surfaces of trails: Water was carefully added to the surface of a water agar plate bearing slugs and slime trails. Slugs and trails floated off the agar. The agar plate was then slowly submerged in a 2-l beaker of water so that the slugs and trails remained floating on the surface of the water. These were picked up by placing a hydrophobic glass slide underneath the floating slugs and trails and carefully raising the slide. The lower surfaces of the trails adhered to the glass so that the upper surfaces remained exposed. Immunolabelling: Air-dried slides were treated overnight at 4°C with an undiluted tissue culture supernatant of the monoclonal antibody MUD50^{9,10}. After three 5-min washes with PBS (0.02 M sodium phosphate-buffered 0.9% saline, pH 7.2), slides were blotted and treated overnight at 4°C with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin diluted 1:100 with PBS. After three more 5-min washes, trails were mounted and examined under a fluorescence phase-contrast microscope. As a control for MUD50, we used MUD3, a spore-specific monoclonal antibody which recognizes no slime component¹⁴; negligible fluorescence resulted (data not shown). Trails fixed in 70% ethanol, 4% formalin in 0.02 M sodium phosphate buffer (pH 7.2) or similarly buffered 2.5% glutaraldehyde also produced acceptable results. Incubation for shorter periods (30 min for each antibody) resulted in less intense fluorescence.



the sheath is less permeable to the monoclonal antibody than the lower layer. Cell prints are not evident in dark-field and are barely discernible in phase-contrast micrographs (Fig. 1*f*). Routine protein stains do not reveal cell prints (data not shown). We therefore estimate that the 'cell print' proteins recognized by MUD50 comprise only a small proportion of total sheath protein.

We investigated the possibility that the two types of pattern seen in the MUD50-treated trail are associated with the two modes of locomotion observed in time-lapse films of migrating slugs. Large slugs 'shuffle' in a pulsatile manner; each slug repeatedly protrudes and lowers its nose. Small slugs (and occasionally large slugs) 'hurdle' in a manner similar to looping caterpillars; hurdling is like shuffling, but the protrusion of the nose is greatly exaggerated—the slug rears and stretches forward, the advanced anterior is then lowered to the substratum and an arch is formed. The rear of the slug follows and the process is then repeated.

Migrating slugs were examined under a polarizing microscope. Between crossed polarizing filters, slugs appear dark against a dark background; when part of a slug is raised above the substratum, that part appears bright because stresses in the sheath tend to align the sheath macromolecules (Fig. 2*d, h*). We matched three series of polarizing light micrographs of migrating slugs with the patterns revealed by MUD50 on the lower surface of their trails. Elliptical patterns were associated with hurdling

(Fig. 2*a-d*) and U-shaped patterns were associated with shuffling (Fig. 2*e-h*).

We envisage that the anterior cells of the slug secrete a substance which passes through the sheath, where it is identified by MUD50 on the lower surface of the trail. The sharpness of the cell prints strongly suggests that anterior cells are stationary on the sheath when the substance is secreted. We propose that the substance is one of a new class of 'traction proteins' which the anterior cells of the slug secrete in order to adhere to the substratum. With its front glued down, the slug contracts to pull its posterior forward through the sheath. Then the nose is protruded and lowered, a new 'zone of adhesion' is formed and the cycle is repeated (Fig. 3).

This scheme for slug locomotion differs from that suggested recently by Odell and Bonner⁸, for these authors claim that there is no interaction between the slug and the substratum during locomotion. In their model, each amoeba within the slug is active and attempts to move anteriorly. The effort with which this attempt is made varies between cells due to a hypothesized chemical gradient. The result is an internal fountain of cells which provides the motive force for slug migration.

In contrast to Odell and Bonner⁸, we view the sheath as more than an interface between the cells of the organism and the environment. Our results clearly demonstrate that some slug cells interact with their ECM and that this interaction is fundamental to slug movement. Characterization of traction proteins

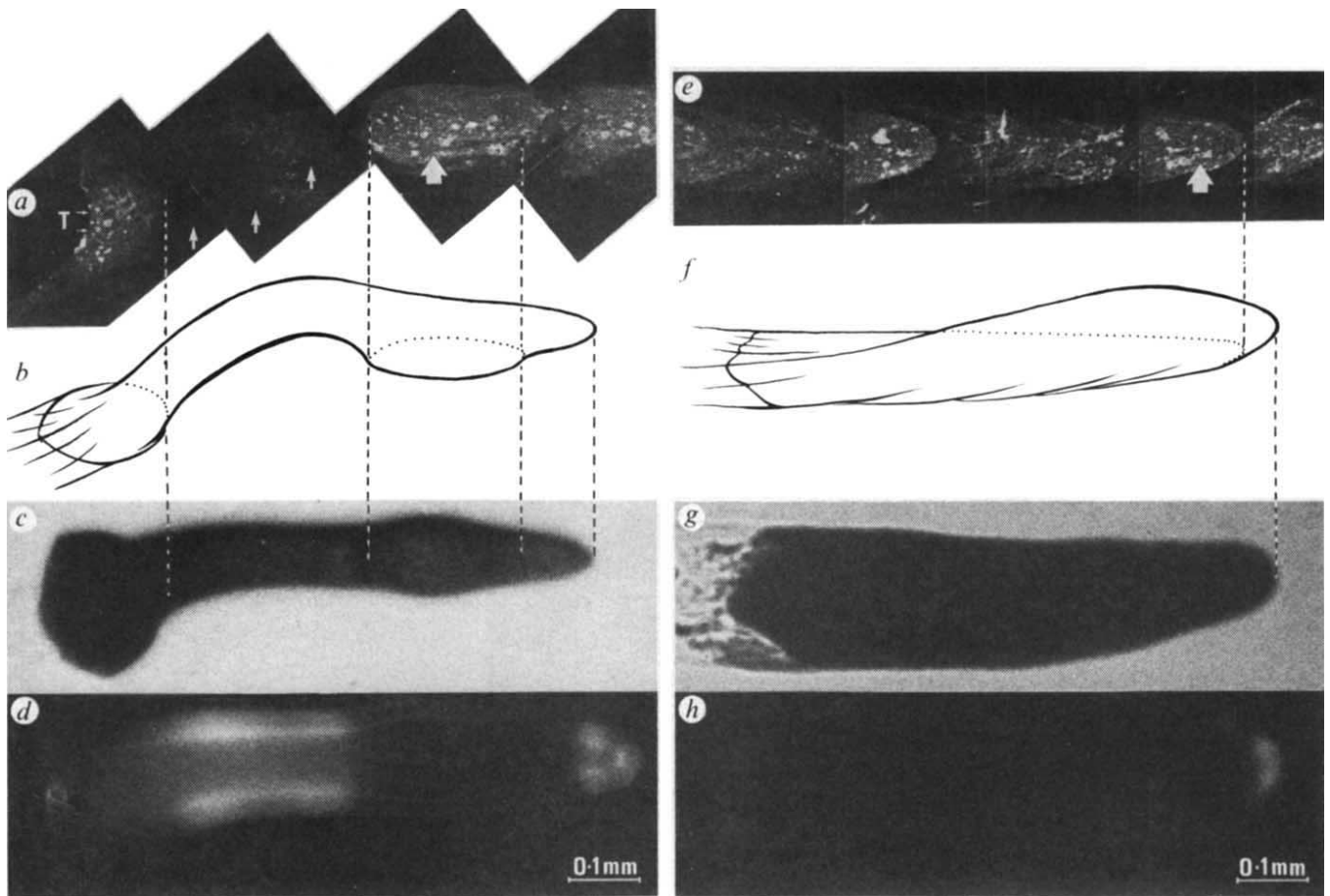


Fig. 2 Correlation of slug movements and elliptical and U-shaped patterns. *a, e*, Segments of a slime trail treated with MUD50 to reveal patterns. The trail in *a* has been torn (T). *b, f*, Illustrations of slug attitude when the trails in *a* and *e* respectively were formed. Dotted lines complete the boundary of the area of contact between the slug and the substratum. *c, g*, Silhouettes of the slug as in *b* and *f*. *d, h*, Polarized light micrographs of the slug taken a few moments after *c* and *g*, respectively. Dashed lines join corresponding regions. At the onset of the experiment (*a-d*): Note the absence of cell prints in the part of the trail which encased the arch (*a*, small arrows). The slug has reared vertically and then arched forward until it has again touched the agar surface (*b*). The slug has then secreted the substance recognized by MUD50 at the elliptical area of contact between the anterior of the slug and the substratum (*a*, large arrow). In cross-section, the arch of the slug is a circle whose diameter is less than the anterior and posterior areas of contact (*c*). The cantilevered nose and the arched rear appear bright above the surface (*d*). Thirty minutes after onset of experiment (*e-h*): Note the sequence of more closely spaced U-shaped patterns formed as the slug has continued to advance (*e*). The nose has been thrust forward as a cantilever (*f*) rather than as an arch. The nose has then been lowered and the substance recognized by MUD50 has been secreted at the U-shaped area of contact between the anterior of the slug and the substratum (*e*, large arrow). The length of the slug (*g*) is approximately the same as in *c*; however, most of the slug is wider because it has flattened. Only the nose appears bright above the surface (*h*).

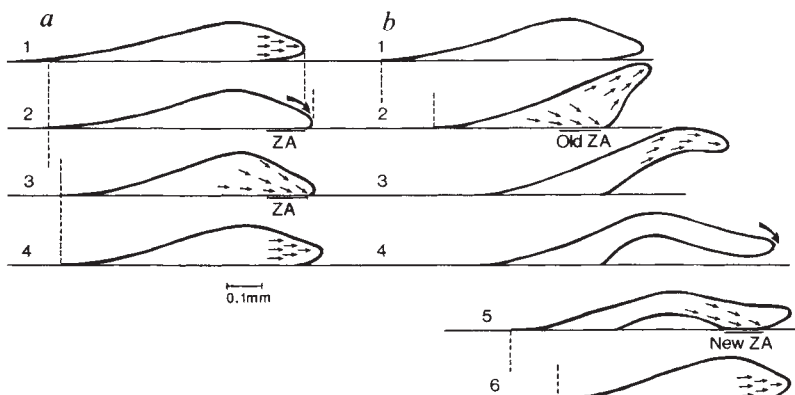


Fig. 3 Slug movement: small arrows show directions of cell motion. *a*, Shuffling: (1) Protrusion of nose; (2) lowering of nose (large arrow) and formation of zone of adhesion (ZA); (3) contraction of slug to advance rear; (4) repetition of process (as in 1). *b*, Hurdling: (1, 2) Advancing tail and rearing upwards; (3) arching forward; (4) lowering of nose to complete arch (large arrow); (5) formation of new zone of adhesion (ZA) and contraction of slug to advance rear; (6) repetition of process (as in 1).

with MUD50 and other monoclonal antibodies is under way and should provide further insight into the biochemistry of coordinated cell movement and the role of ECM in this simple multicellular organism.

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1. Boucrot, J. C., Darribère, T., Boulekbache, H. & Thiery, J. P. *Nature* **307**, 364-367 (1984).
2. Le Douarin, N. M. *Cell* **38**, 353-360 (1984).
3. Raper, K. B. *J. Elisha Mitchell scient. Soc.* **59**, 241-282 (1940).
4. Hohl, H. R. & Jehli, J. *Archs Microbiol.* **92**, 179-187 (1973).
5. Smith, E. & Williams, K. L. *FEMS microbiol. Lett.* **6**, 119-122 (1979).
6. Freeze, H. & Loomis, W. F. *Devl Biol.* **56**, 184-194 (1977).
7. Garrod, D. R. *J. Cell Sci.* **4**, 781-798 (1969).
8. Odell, G. M. & Bonner, J. T. *Phil. Trans. R. Soc. B* (in the press).
9. Grant, W. N. & Williams, K. L. *EMBO J.* **2**, 935-940 (1983).
10. Grant, W. N., Welker, D. L. & Williams, K. L. *Molec. cell. Biol.* **5**, 2559-2566 (1985).
11. Robson, G. E. & Williams, K. L. *Curr. Genet.* **1**, 229-232 (1980).
12. Fisher, P. R., Smith, E. & Williams, K. L. *Cell* **23**, 799-807 (1981).
13. Krefft, M., Voet, L., Gregg, J. H., Mairhofer, H. & Williams, K. L. *EMBO J.* **3**, 201-206 (1984).
14. Voet, L., Krefft, M., Bruderlein, M. & Williams, K. L. *J. Cell Sci.* **75**, 423-435 (1985).

Postsynaptic hyperpolarization during conditioning reversibly blocks induction of long-term potentiation

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Activity-induced changes in the efficacy of synaptic transmission between neurones are central to several prominent theories of learning¹⁻⁴. In both *in vivo* and *in vitro* preparations of the hippocampus, a conditioning high-frequency stimulus delivered to afferent fibres results in a long-term potentiation of synaptic transmission at those inputs⁵⁻⁸. Evidence has been provided supporting both presynaptic^{5,9-11} and postsynaptic¹²⁻¹⁴ sites as loci where critical events occur in the development of potentiation. In this study we report that long-term potentiation is reversibly blocked by intracellular injection of hyperpolarizing current in the postsynaptic cell during the conditioning high-frequency stimulus, suggesting the involvement of a voltage-dependent postsynaptic mechanism.

Rat hippocampal slices were prepared and maintained *in vitro* as previously described^{15,16}. Each slice was used for only one experiment. Two intracellular electrodes filled with 3M KCl (80-120 MΩ) were placed in the CA1 cell body layer, and a bipolar electrode was placed in the CA2 dendritic area close to the cell body layer, as shown in Fig. 1a. An intracellular impalement was judged to be satisfactory if the resting potential was >55 mV and action potentials were >75 mV. Once a satisfactory impalement was obtained by both electrodes, test stimuli were delivered through the bipolar electrode at a rate of 0.3 Hz. During this and all subsequent test periods, a constant 1 nA of hyperpolarizing current was passed through each intracellular electrode to increase the amplitude of the excitatory postsynaptic potentials (e.p.s.ps). The stimulation current was adjusted to a level at which the sizes of the e.p.s.ps were sub-threshold for spike generation. If the e.p.s.p. responses proved stable for 10 min, the hyperpolarizing current was increased to 3 nA in one cell (test cell) and reduced to zero in the other cell (control

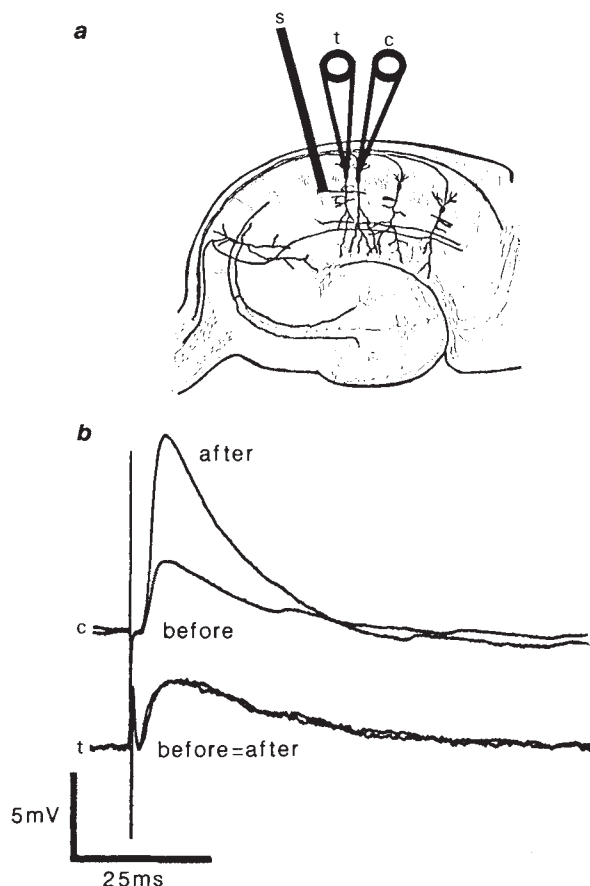


Fig. 1 *a*, Diagram of a hippocampal slice showing the location of the bipolar extracellular stimulating electrode (s), the intracellular electrode in the control cell (c), and the intracellular electrode in the test cell (t). (Adapted from Ramón y Cajal²⁰.) *b*, The upper traces show an intracellular recording from a control cell (c) which did not receive any current passage during the conditioning HFS. Each trace is the average of five successive responses 4 s apart recorded immediately before and 10 minutes after the conditioning HFS. Lower traces in *b* show a simultaneous intracellular recording from a nearby test cell (t) which received passage of a 3-nA hyperpolarizing current during the HFS. Averaged responses were recorded immediately before and 10 min after the HFS. The control cell's response was potentiated by the HFS whereas the e.p.s.p. of the test cell was not.

cell). A conditioning high-frequency stimulus (HFS) consisting of a 5-s 30-Hz burst of pulses was then delivered through the bipolar electrode. Subsequently, intracellular passage of current was restored to pre-conditioning levels and responses to test stimuli were averaged and recorded for the next 15-20 min. Data were accepted from experiments in which, following the conditioning stimulus, (1) stable intracellular recordings were maintained in both test and experimental cells for 15 min and (2) the control cell showed a sustained enhanced synaptic response to test stimuli which exceeded the baseline response by at least 20%. Six experiments satisfied these criteria. To test whether the transient hyperpolarization caused an irreversible change in the test cells' ability to undergo long-term potentiation (LTP), test cells which showed stable recordings for 15 min following the test period (3 of 6) and four other cells from separate slices were injected with 3 nA of hyperpolarizing current followed by (but not coincident with) a conditioning HFS. Responses to test stimuli were then averaged and recorded for 15 min.

The results reveal that the e.p.s.ps of the (test) cells which had been hyperpolarized during the HFS showed no significant increase in amplitude (Figs 1, 2). When those test cells (or cells

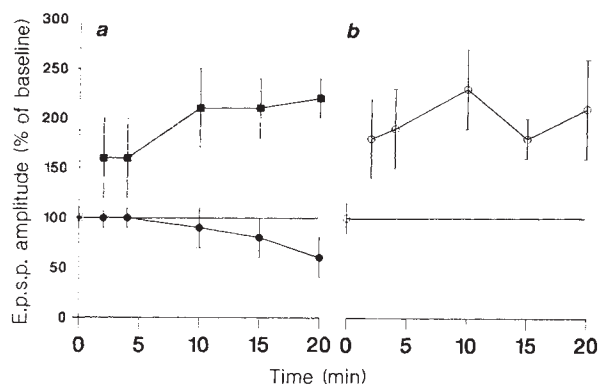


Fig. 2 Plot of the amplitude of e.p.s.ps following an HFS. The x axis is time in minutes following the conditioning stimulus. On the y axis, 100% represents the mean response before delivery of a conditioning stimulus. *a*, Hyperpolarization during the conditioning HFS prevents LTP. Each point in the curve with filled squares represents the mean response ($\pm$ s.e.m.) of a (control) cell which was delivered a conditioning HFS at time 0 in the absence of hyperpolarizing current. Each point in the curve with filled circles represents the mean response ($\pm$ s.e.m.) measured simultaneously from a (test) cell in which 3 nA of hyperpolarizing current was passed during the conditioning HFS ($n = 6$ for $t = 0-15$ min, $n = 2$ for $t = 20$ min). The symbol at time zero represents the mean response ($\pm$ s.e.m.) of all cells before the conditioning stimulus. Student's *t*-test shows significant difference between test and control groups: $t = 10$ min, $P < 0.025$; $t = 15$ min, $P < 0.001$; $t = 20$ min, $P < 0.001$. *b*, Hyperpolarization prior to conditioning HFS does not prevent LTP. Cells were hyperpolarized by a 3-nA passage of current and subsequently delivered a conditioning HFS. Each point represents the mean response ($\pm$ s.e.m.) ($n = 7$ for $t = 0-15$ min, 3 of 6 test cells + 4 randomly chosen cells from other slices; $n = 3$ for $t = 20$, 3 of the 4 randomly chosen cells).

which had been hyperpolarized by the passage of 3 nA of current) were subsequently delivered an HFS in the absence of hyperpolarizing current, there was a potentiation of the response equivalent to that observed in the control cells. The ability to undergo LTP and the absence of a change in input resistance ($<5\%$) demonstrates the continued health of test cells following a transient 3-nA hyperpolarizing current passage. In agreement with previous intracellular studies on LTP^{17,18}, we observed no significant change ($<5\%$) in input resistance in the control cells.

Our results contradict those obtained by Wigström *et al.*¹⁹, who found that the induction of LTP was apparently unaffected by hyperpolarization of the postsynaptic cell during a conditioning stimulus. Using a conditioning paradigm like theirs, we too were unable to block the induction of LTP (data not shown). The experimental paradigm of the present study differed from theirs in four significant ways: (1) we recorded intracellularly from two postsynaptic cells simultaneously and stimulated only one input tract, rather than stimulating two input tracts and recording from one postsynaptic cell; (2) we intentionally stimulated a limited sub-population of input fibres which, due to the general laminar organization of the hippocampus^{20,21}, were presumed to make synapses close to the cell bodies; (3) we passed 3 nA of hyperpolarizing current during the HFS instead of 1 nA; and (4) we used a 30-Hz rather than a 50-100-Hz conditioning HFS. The first of these precautions was taken to avoid any postsynaptic associative interactions¹⁷ between different inputs, whereas the last three were taken to increase the reliability of hyperpolarizing the postsynaptic membrane at the regions where the afferent synapses were located. In particular, we suspect that a greater amplitude intracellular current passage and a slower conditioning stimulus used in our experimental paradigm allowed re-hyperpolarization of the postsynaptic membrane

between transmitter-induced postsynaptic conductances. Since a transmitter-induced synaptic conductance may still be at almost 20% of its maximum 10 ms after its onset²², a 100-Hz conditioning stimulus such as the one used by Wigström *et al.*, could yield overlapping conductances and could drive the postsynaptic membrane toward the reversal potential of the synaptic conductance. This would be largely unaffected by hyperpolarizing current being driven through the cell body and thus mechanisms contingent on sub-synaptic membrane depolarization could still be activated. A 30-Hz conditioning stimulus such as the one we used, on the other hand, would allow the transmitter-induced conductance to decay between individual synaptic conductances and thus the hyperpolarizing current being passed through the soma may keep the sub-synaptic membrane potential polarized. For these reasons, it is likely that the discrepancy between our results and those presented by Wigström *et al.* are due to the differences in experimental paradigms.

Here we have shown that hyperpolarization of the postsynaptic cell during the conditioning stimulus reversibly blocks the development of LTP. This implies that voltage-dependent postsynaptic mechanisms are necessary for the development of LTP. Previous studies have shown that LTP is specific to the conditioned input tracts⁸, and that intracellular injection of the calcium chelator EGTA into the postsynaptic cell prevents the development of LTP¹². Together, these results are consistent with the hypothesis that depolarization of the postsynaptic membrane during the conditioning HFS opens local voltage-sensitive calcium channels, and that the resulting local increase in intracellular calcium concentration triggers a mechanism which leads to the eventual development of LTP.

The result we report may shed light on two phenomena previously described in the literature. The first concerns the associative nature of LTP, whereby a strong conditioning input coincident with a weak input leads to potentiation of the response to the weak input¹⁷. Our findings are consistent with the notion that the postsynaptic membrane depolarization induced by the strong input is electrotonically (or actively) propagated to the site of the weak input and that this summed depolarization is necessary for the development of LTP of the weak input²³. Second, our result complements previous reports which implicate inhibition in controlling LTP^{24,25}. One may speculate that local inhibitory activity regulates the potentiation of synaptic transmission by preventing the postsynaptic membrane potential from reaching a critical level necessary to trigger the mechanisms responsible for LTP.

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- Hebb, D. O. *Organization of Behavior* (Wiley, New York, 1949).
- Marr, D. J. *Physiol. Lond.* **202**, 437-470 (1969).
- Albus, J. S. *Math. Biosci.* **10**, 25-61 (1971).
- Stent, G. S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 997-1001 (1973).
- Bliss, T. V. P. & Lomo, T. J. *Physiol. Lond.* **232**, 331-356 (1973).
- Bliss, T. V. P. & Gardner-Medwin, A. R. J. *Physiol. Lond.* **232**, 357-374 (1973).
- Schwartzkroin, P. A. & Wester, K. *Brain Res.* **89**, 107-119 (1975).
- Andersen, P., Sundberg, S. H., Sveen, O. & Wigström, H. *Nature* **266**, 736-737 (1977).
- Dolphin, A. C., Errington, M. L. & Bliss, T. V. P. *Nature* **297**, 496-498 (1982).
- Andersen, P., Sundberg, S. H., Sveen, O., Swan, S. W. & Wigström, H. *J. Physiol., Lond.* **302**, 463-482 (1980).
- McNaughton, B. L., Douglas, R. M. & Goddard, G. V. *Brain Res.* **157**, 277-293 (1978).
- Lynch, G., Larson, J., Kelso, S., Barrionuevo, G. & Schottler, F. *Nature* **305**, 719-721 (1983).
- Dunwiddie, T. & Lynch, G. J. *Physiol. Lond.* **276**, 353-367 (1978).
- Baudry, M., Bundman, M. C., Smith, E. K. & Lynch, G. *Science* **212**, 937-938 (1981).
- Nicoll, R. A. & Alger, B. E. *J. Neurosci. Meth.* **4**, 153-156 (1981).
- Alger, B. E. & Nicoll, R. A. J. *Physiol. Lond.* **328**, 105-123 (1982).
- Barrionuevo, G. & Brown, T. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7347-7351 (1983).
- Barrionuevo, G. & Brown, T. H. *Soc. Neurosci. Abstr.* **10**, 78 (1984).
- Wigström, H., McNaughton, B. L. & Barnes, C. A. *Brain Res.* **233**, 195-199 (1982).
- Ramón y Cajal, S. *Histologie du système nerveux de l'Homme et des Vertébrés* (Maloine, Paris, 1911).
- Swanson, L. W., Wyss, J. M. & Cowan, W. M. J. *comp. Neurol.* **181**, 681-716 (1978).
- Brown, T. H. & Johnston, D. J. *Neurophysiol.* **50**, 487-507 (1983).
- Kelso, S. R. & Brown, T. H. *Soc. Neurosci. Abstr.* **11**, 780 (1985).
- Douglas, R. M., Goddard, G. V. & Riives, M. *Brain Res.* **240**, 259-272 (1982).
- Wigström, H. & Gustafsson, B. *Nature* **301**, 603-604 (1983).

Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage

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Many observations suggest the presence of transmembrane linkages between the cytoskeleton and the extracellular matrix. In fibroblasts both light and electron microscopic observations reveal a co-alignment between actin filaments at the cell surface and extracellular fibronectin¹⁻³. These associations are seen at sites of cell matrix interaction, frequently along stress fibres and sometimes where these bundles of microfilaments terminate at adhesion plaques (focal contacts). Non-morphological evidence also indicates a functional linkage between the cytoskeleton and extracellular matrix. Addition of fibronectin to transformed cells induces flattening of the cells and a reorganization of the actin cytoskeleton, with the concomitant appearance of arrays of stress fibres⁴⁻⁶. Conversely, disruption of the actin cytoskeleton by treatment with cytochalasin B leads to release of fibronectin from the cell surface⁷. As yet, there is no detailed knowledge of the molecules involved in this transmembrane linkage, although several proteins have been suggested as candidates in the chain of attachment between bundles of actin filaments and the cytoplasmic face of the plasma membrane: these include vinculin⁸, α -actinin⁹ and talin¹⁰, each one having been identified at regions where bundles of actin filaments interact with the plasma membrane and underlying cell-surface fibronectin¹⁰⁻¹³. Recently, the cell-substrate attachment (CSAT) antigen¹⁴ has been identified as a plasma membrane receptor for fibronectin¹⁵, raising the possibility that this glycoprotein complex may serve as a bridge between fibronectin and one or more of the underlying cytoskeletal components mentioned. Here we have investigated the interaction of the purified CSAT antigen with these cytoskeletal components, and we demonstrate an interaction specifically between the CSAT antigen and talin.

The CSAT antigen is an integral membrane glycoprotein complex recognized by the CSAT monoclonal antibody¹⁶. This antibody inhibits the adhesion and perturbs the morphology of several different avian cell types including fibroblasts, muscle cells and neurones^{17,18}. The antigen consists of three distinct glycoproteins of relative molecular mass (M_r) ~140,000 (140K) on SDS gels¹⁶. The antigen co-localizes extensively with fibronectin along portions of stress fibres and is found at their termini¹⁹. Similar observations have been made using another monoclonal antibody, JG-22, directed against the same antigen²⁰⁻²². Recently, the purified antigen has been shown to bind to fibronectin in a physiologically significant manner¹⁵, being inhibited from binding by the fibronectin tetrapeptide (RGDS) that inhibits adhesion of cells to fibronectin²³. Glycoproteins of M_r ~140K have also been implicated in the adhesion of mammalian cells to fibronectin^{24,25}. This identification of the CSAT antigen as a fibronectin receptor has prompted further studies to determine whether it may also function as a link between fibronectin and cytoskeletal components.

Interaction between the CSAT antigen and vinculin, talin or α -actinin was assayed initially using Western blotting. The CSAT antigen was radio-iodinated and used to overlay nitrocellulose strips onto which these other proteins had been elec-

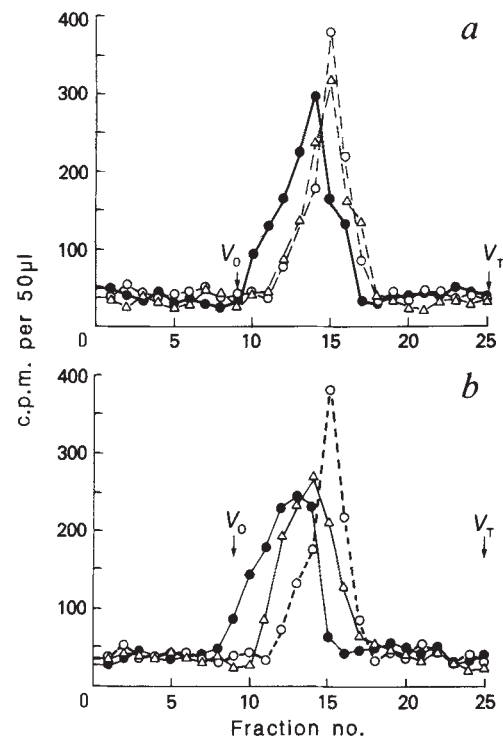


Fig. 1 *a*, Profile of elution of CSAT antigen from gel-filtration columns pre-equilibrated with talin. The CSAT antigen was passed through a column equilibrated with 900 $\mu\text{g ml}^{-1}$ vinculin (Δ) or 500 $\mu\text{g ml}^{-1}$ talin ($\bullet$). $\circ$, CSAT antigen alone. *b*, Profile of elution of CSAT antigen from gel-filtration columns pre-loaded with: 800 $\mu\text{g ml}^{-1}$ fibronectin and 200 $\mu\text{g ml}^{-1}$ talin ($\bullet$) or 800 $\mu\text{g ml}^{-1}$ fibronectin, 200 $\mu\text{g ml}^{-1}$ talin and 1 mg ml^{-1} of the cell-binding tetrapeptide (RGDS) from fibronectin (Peninsula Laboratories, Belmont, California) (Δ). $\circ$, CSAT antigen alone.

Methods. Gel filtration was performed using Ultrogel AcA22 (LKB) in a column of 0.2×30 cm bed volume. The column was first pre-loaded with the appropriate ligand in buffer B (20 mM NaCl, 20 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β -mercaptoethanol, 0.1% Nonidet P-40 (NP40), pH 7.6). Similar results were obtained using TNC buffer (0.01 M Tris-acetic acid, 0.5 mM CaCl_2 , 0.15 M NaCl, 0.1% NP40, pH 8.0). The ligands and CSAT antigen (2.2×10^5 mCi mmol^{-1}) were incubated for 30 min before loading onto the ligand-pre-equilibrated column¹⁵. Fractions (60 μl) were collected and 50 μl of each fraction were counted; V_0 and V_T , void and included volumes, respectively. The CSAT antigen was purified from ^{35}S -methionine-labelled chicken tendon fibroblasts using immunoaffinity chromatography with the CSAT monoclonal antibody as the immunoabsorbent^{15,16}. The cytoskeletal proteins talin, vinculin and α -actinin used in this and the other experiments were purified from chicken gizzard smooth muscle essentially as described previously^{10,30}.

trophoretically transferred from SDS gels. No significant binding was detected in this or in the reciprocal experiment, when vinculin, talin and α -actinin were used as radiolabelled probes. Similarly, no binding was observed in sedimentation experiments in which the CSAT antigen was mixed individually with the proteins prior to sucrose density gradient centrifugation. The lack of detectable binding in the latter assay might have been caused by rapid dissociation of receptor-ligand complexes, therefore we used the technique of equilibrium gel filtration: in this method a gel filtration column is pre-equilibrated with the ligand to be assayed (talin, vinculin or α -actinin). The ligands and CSAT antigen (labelled with ^{35}S -methionine) were then incubated for 30 min before loading onto the column. (Further details of the assay are presented elsewhere¹⁵.) The method ensures receptor occupancy for rapidly dissociating receptor-ligand complexes.

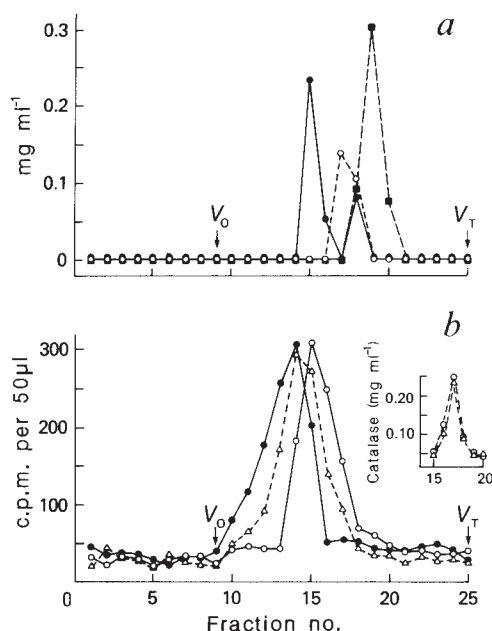


Fig. 2 *a*, Gel-filtration elution profiles (mg ml⁻¹) of talin (○), vinculin (■), and talin-vinculin complex (●), using Ultrogel AcA22. Equal amounts of talin and vinculin were incubated together on ice for 2 h, brought to room temperature, then analysed by gel filtration. Protein concentrations were determined by the method of Lowry *et al.*³¹. *b*, Profile of elution of the CSAT antigen from gel-filtration columns pre-equilibrated with 200 µg ml⁻¹ talin (△), 200 µg ml⁻¹ vinculin (○), or 200 µg ml⁻¹ talin plus 200 µg ml⁻¹ vinculin (●). Inset, the elution positions (mg ml⁻¹) of a catalase (20 µg) Stokes radius standard when added to either the CSAT antigen-talin mixture (△) or the CSAT antigen-vinculin mixture (○) before gel filtration. Protein concentrations were determined by the method of Lowry *et al.*³¹, and the background values due to ligand subtracted. Gel filtration was performed as described in Fig. 1 legend.

Figure 1a shows the elution profile of the detergent-solubilized CSAT antigen during gel filtration on Ultrogel AcA22 (LKB). The antigen is seen as a single peak eluting between fractions 13 and 18. When the column was first pre-loaded with talin at concentrations >25 µg ml⁻¹, the elution profile shifted towards a higher effective Stokes radius, revealing an interaction between the CSAT antigen and talin. Such shifts were not seen when the mixtures of the CSAT antigen and talin were added to a column that had not been pre-loaded with talin, indicating that the complex formed between the two proteins tends to dissociate rapidly on the timescale of the molecular sieving. The magnitude of the shift increased with increasing talin concentration, reaching a plateau at ~300 µg ml⁻¹. The half-maximal shift was at ~150 µg ml⁻¹, which corresponds to a dissociation constant, K_d , of $\sim 7 \times 10^{-7}$ M. The elution profile of the antigen during gel filtration in a column pre-equilibrated with either 900 µg ml⁻¹ vinculin (Fig. 1a) or 2 mg ml⁻¹ α -actinin (not shown) was identical to those seen with columns that had not been pre-equilibrated with a ligand. The 'talin shift' was observed in each of 21 separate determinations using 3 different antigen and talin preparations. The elution profile of catalase, a Stokes radius standard, when added to the CSAT antigen-vinculin and CSAT antigen-talin mixtures prior to gel filtration, was invariant (see Fig. 2 inset), as reported previously for fibronectin and laminin¹⁵.

The physiological significance of the interaction between the CSAT antigen and fibronectin was established¹⁵ by its inhibition using the fibronectin cell-binding tetrapeptide. We have included this same tetrapeptide with talin during equilibrium gel filtration and observed no effect on the formation of the talin-CSAT

antigen complex. This result indicates that talin and fibronectin bind to separate sites on the CSAT antigen; this has been confirmed in further experiments in which it was found that when both fibronectin and talin were present during gel filtration, there was a larger shift in the CSAT antigen elution profile than occurred in the presence of either ligand alone (Fig. 1b). Moreover, the presence of the fibronectin cell-binding tetrapeptide diminished the magnitude of this shift to one similar to that seen with talin alone (Fig. 1b).

It has been demonstrated previously that vinculin and talin form a complex of moderately high affinity²⁶. This complex can also be seen by gel filtration (Fig. 2a): 80% of the total protein migrates in the complex at a higher effective Stokes radius. We have assayed the interactions of the complex with the purified CSAT antigen. Pre-loading of the gel filtration column with the talin-vinculin complex also shifted the elution profile of the antigen (Fig. 2b); but the magnitude of this shift was consistently larger than that observed with talin alone. The simultaneous interaction of talin with both the CSAT antigen and vinculin indicates that talin has separate binding sites for the two molecules.

It has been shown that talin is highly susceptible to proteolysis, resulting in the cleavage of the molecule into two domains of $M_r \sim 190K$ and $46K$ (refs 27, 28). In some systems, such as platelet activation, this proteolysis appears to be physiologically significant and involves a calcium-dependent protease^{27,28}. Previous work has shown that the larger talin domain binds vinculin²⁹, and we queried whether proteolysis would separate the vinculin-binding domain from the CSAT antigen-binding domain. When the purified 190K fragment of talin was substituted for talin in the equilibrium gel filtration assays, however, it behaved like intact talin and induced an indistinguishable shift in the elution profile of the CSAT antigen (data not shown), indicating that this large talin domain contains binding sites for both vinculin and the CSAT antigen.

The transmembrane link between fibronectin and the actin cytoskeleton has been elusive. Several observations have implicated the CSAT antigen as a component in this linkage: (1) the CSAT monoclonal antibody inhibits adhesion and perturbs the morphology of several different cell types^{14,17,18}, (2) the antigen is localized in regions of cell adhesion and putative transmembrane coupling¹⁹, (3) the antigen appears to span the membrane, as suggested by the existence of two classes of monoclonal antibodies that bind to either intact or saponin-permeabilized cells (C.B., C. Decker and A.H., unpublished observations), (4) the antigen is an oligomeric complex of three integral membrane glycoproteins which serves as a cell-surface receptor for fibronectin and other extracellular matrix molecules^{15,16}. We have now shown that the CSAT antigen has a binding domain for talin, a protein associated with the actin cytoskeleton. Although talin has been shown to bind vinculin, it remains to be established how talin or the talin-vinculin complex interacts with actin. We conclude that the CSAT antigen appears to function in transmembrane linkage as a dual receptor possessing at least two distinct classes of binding domains: one for extracellular molecules like fibronectin and the other for cytoskeletal proteins such as talin.

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- Hynes, R. O. & Destree, A. T. *Cell* **15**, 875-886 (1978).
- Heggenes, M. H., Ash, J. F. & Singer, S. J. *Ann. N.Y. Acad. Sci.* **312**, 414-417 (1978).
- Singer, I. I. *Cell* **16**, 675-685 (1979).
- Yamada, K. M., Ohanian, S. H. & Pastan, I. *Cell* **9**, 241-245 (1976).
- Ali, I. U., Mautner, V., Lanza, R. & Hynes, R. O. *Cell* **11**, 115-126 (1977).
- Willingham, M. C., Yamada, K. M., Yamada, S. S., Pouyssegur, J. & Pastan, I. *Cell* **10**, 375-380 (1977).
- Ali, I. U. & Hynes, R. O. *Biochim. biophys. Acta* **471**, 16-24 (1977).
- Geiger, B. *Cell* **18**, 193-205 (1979).

9. Lazarides, E. & Burridge, K. *Cell* **6**, 289–298 (1975).
10. Burridge, K. & Connell, L. *J. Cell Biol.* **97**, 359–367 (1983).
11. Burridge, K. & Feramisco, J. R. *Cell* **19**, 587–595 (1980).
12. Singer, I. I. & Paradiso, P. R. *Cell* **24**, 481–492 (1981).
13. Chen, W. T. & Singer, S. J. *J. Cell Biol.* **95**, 205–233 (1982).
14. Neff, N. T. *et al.* *J. Cell Biol.* **95**, 654–666 (1982).
15. Horwitz, A., Duggan, K., Greggs, R., Decker, C. & Buck, C. *J. Cell Biol.* **101**, 2134–2144 (1985).
16. Knudsen, K., Horwitz, A. & Buck, C. *Exp. Cell Res.* **157**, 218–226 (1985).
17. Decker, C., Greggs, R., Duggan, K., Stubbs, J. & Horwitz, A. *J. Cell Biol.* **99**, 1398–1404 (1984).
18. Bozyczko, D. & Horwitz, A. *J. Neurosci.* (in the press).
19. Damsky, C., Knudsen, K., Bradley, D., Buck, C. & Horwitz, A. *J. Cell Biol.* **100**, 1528–1539 (1985).
20. Greve, J. M. & Gottlieb, D. I. *J. cell. Biochem.* **18**, 221–230 (1982).
21. Chen, W. T., Hasegawa, E., Hasegawa, T., Weinstock, C. & Yamada, K. M. *J. Cell Biol.* **100**, 1103–1114 (1985).
22. Hasegawa, T., Hasegawa, E., Chen, W. T. & Yamada, K. M. *J. cell. Biochem.* **28**, 307–318 (1985).
23. Pierschbacher, M. & Ruoslahti, E. *Nature* **309**, 30–33 (1984).
24. Brown, P. J. & Juliano, R. *Science* **228**, 1448–1451 (1985).
25. Pytela, R., Pierschbacher, M. & Ruoslahti, E. *Cell* **40**, 191–198 (1985).
26. Burridge, K. & Mangeat, P. H. *Nature* **308**, 744–745 (1984).
27. O'Halloran, T. O., Beckerele, M. C. & Burridge, K. *Nature* **317**, 449–451 (1985).
28. Beckerele, M. C., O'Halloran, T. O. & Burridge, K. *J. cell. Biochem.* (in the press).
29. O'Halloran, T. O. & Burridge, K. *Biochim. biophys. Acta* (in the press).
30. Feramisco, J. R. & Burridge, K. *J. biol. Chem.* **255**, 1194–1199 (1980).
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).

Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs

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The basic principles of the architecture of many viral protein shells have been successfully established from electron microscopy and X-ray data^{1–4}, but enveloped viruses have been more difficult to study because they resist crystallization and are easily deformed when prepared for electron microscopy. To avoid the limitations of conventional techniques when applied to enveloped viruses, we have used a cryo-electron microscopy method in which unfixed and unstained viruses are observed in an unsupported thin layer of vitrified suspension^{5,6}. Because of electron beam damage, the many different views required for high-resolution three-dimensional reconstruction cannot be obtained from a tilt series of the same particle. The images of many differently oriented viruses are combined using a novel reconstruction method, 'reconstruction by optimized series expansion' (ROSE)⁷. The structure of the envelope of Semliki Forest virus has been reconstructed to 3.5-nm resolution. The $T = 4$ geometry of the surface lattice, the shape of the trimeric spikes and their arrangement on the lipid bilayer are visualized.

Semliki Forest virus (SFV) is one of the simplest icosahedral enveloped RNA viruses^{8–11}. The centre of the virus consists of the nucleocapsid, surrounded by the lipid bilayer at an average radius of 23 nm. The spike proteins protruding from the lipid bilayer are each formed by three polypeptide chains with a total relative molecular mass of 100,000. They extend to an outer radius of ~32 nm.

Figure 1 shows a focus series of SFV in a thin layer of vitrified buffer solution. The visualized aspects of the virus depend strongly on the focus. This is because, unlike in conventionally stained specimens, the contrast is essentially due to phase contrast¹². The focus values of the four micrographs in the series have been chosen to give an optimal coverage of the information contained in the specimen. Coarse features such as the triangulation

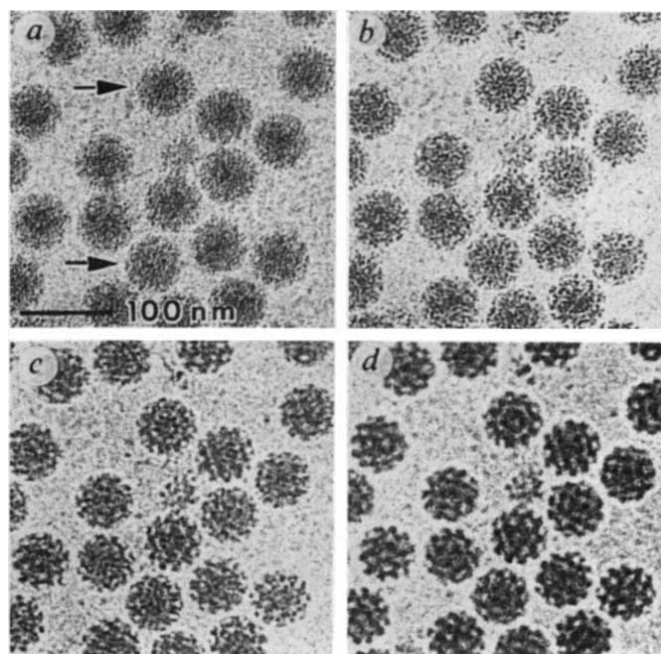


Fig. 1 Focus series of a thin vitrified unsupported layer of unfixed and unstained Semliki Forest virus suspension. The specimen has been prepared by the perforated grid method⁵: a drop of suspension was applied to a copper grid supporting a perforated carbon film. Most of the drop was removed with blotting paper and the grid was immediately immersed in undercooled liquid ethane. The grid, always kept below -160°C , was transferred and observed in a Philips 400 electron microscope working at 80 kV and equipped with the cold stage PW6591/100. The micrographs were recorded at an electron magnification of $\times 33,000$ and the electron dose was 1,000 electrons per nm^2 per micrograph. Micrographs *a–d* are underfocused by 1.5, 3, 6 and 11 μm respectively. These values have been chosen so that the first zero of the transfer function of one micrograph corresponds to the first maximum of the preceding one (see ref. 6, Fig. 5, for illustration). The first maximum corresponds to 3.5 nm in *a* and to 9.5 nm in *d*. In these conditions the four micrographs together retrieve at least 70% of the information in the spatial frequency band corresponding to 3–12 nm. The arrows in *a* point towards particles where the lipid bilayer is visible.

number $T = 4$ of the icosahedral shell are best observed in strongly underfocused images (Fig. 1*d*; see also refs 5, 6). Fine details, such as the lipid bilayer, are visible close to focus (Fig. 1*a*, arrows).

Using the series shown in Fig. 1, a three-dimensional reconstruction of the virus has been obtained with ROSE⁷ (S.W.P. and R.H.V., manuscript in preparation). Various representations of the reconstruction at different resolution are shown in Figs 2–4. The surface of the virus, consisting of 80 spikes, is arranged in a $T = 4$ surface lattice. The concept of quasi-equivalence¹ applied to this geometry requires that each spike be a trimer of spike proteins (that is, 3×3 polypeptide chains). The reconstruction shows that the spikes are indeed triangular. The total number of spike proteins should therefore be 240. This implies that the total mass of the virus should be higher than the value obtained by several direct mass measurement methods^{13,14}. As can be seen from the reconstruction, the suggestion that the virus has a triangulation number $T = 3$ for resolving the discrepancy is ruled out. Another more subtle geometrical prediction is that peripentonal spikes must have a different conformation from those of the 3-fold axes¹⁵. This prediction is supported by the reconstruction in that the peripentonal spikes have a concave surface whereas those centred on the 3-fold axes are convex (Fig. 2).

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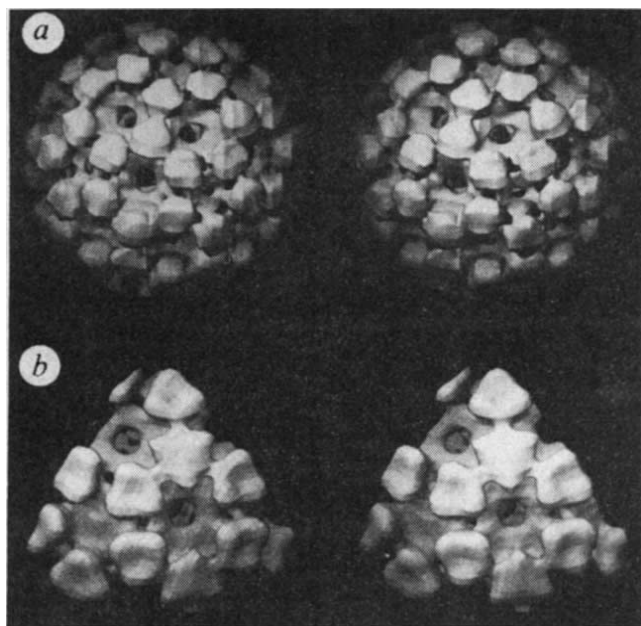


Fig. 2 Stereo view of the SFV reconstruction using ROSE⁷ and the micrograph series shown in Fig. 1. The complete virus at 4.5-nm resolution is shown in *a* whereas *b* shows one octant of the virus at 3.5-nm resolution. ROSE is based on a truncated expansion of the electron density in a complete orthonormal set of basis functions in spherical polar coordinates. These functions are their own Fourier transforms, and rotations of the coordinate system can be expressed using rotation operators for spherical harmonics¹⁹. Selection rules have been used for the expansion coefficients to impose icosahedral symmetry. These coefficients and the Euler angles defining the relative orientation of each particle were simultaneously optimized taking into account the images of 21 randomly selected particles. Eight particles were selected for the final reconstructions shown in Figs 2–4. The resolution has been estimated using point spread functions. The display of the figure has been produced by a modified version of the shadowing program of G. Vigers. The density level of the display corresponds to a volume of membrane protein which is about 1.5 times that estimated from dry molecular mass.

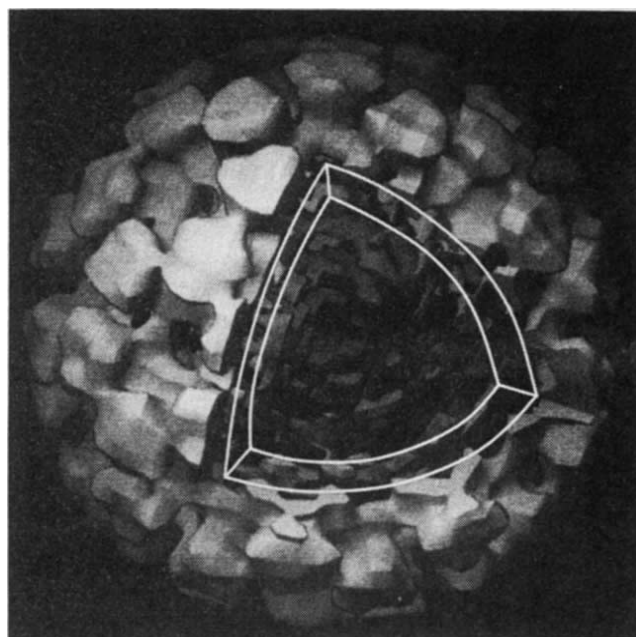


Fig. 3 The same model as in Fig. 2*b*, but with an octant removed in order to show the interior. Solid white lines have been drawn at radii corresponding to 21 and 25 nm. These correspond to the putative positions of the membrane as indicated in Fig. 1*a*.

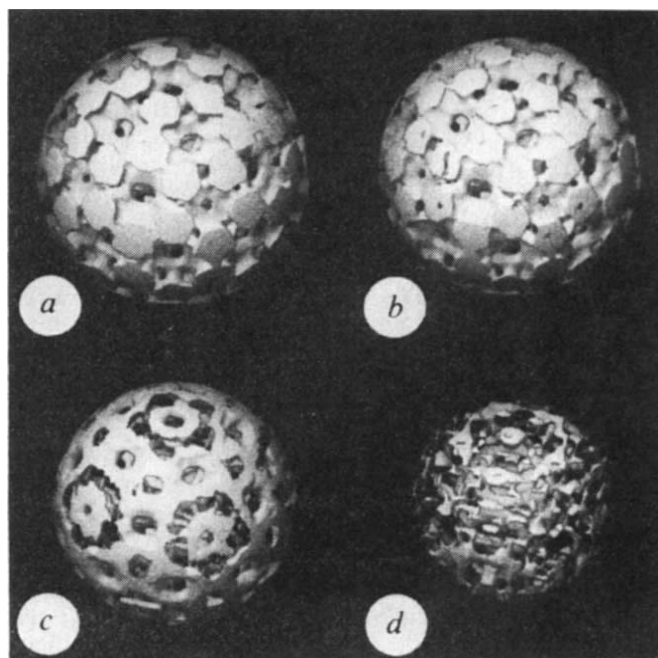


Fig. 4 Same model as in Fig. 2*b*. The outer part of the model has been 'peeled' down to radii corresponding to 31, 29.5, 27.5 and 24 nm in *a-d*, respectively.

The lipid membrane is revealed on radial density plots of the reconstruction as a low-density region at a radius of 23 nm (not shown). It is also visible in Fig. 3 and in Fig. 4*d* where it corresponds to a low-density surface with no well-defined structure. Figure 3 also shows that each spike has the shape of an upside-down mushroom. The foot forms the outermost part of the spike. At that level the spikes are well separated with considerable free space. At a radius smaller than 29 nm, they spread, forming an ~3-nm-thick dense layer covering nearly all of the lipid bilayer. Each spike also seems to induce a local deformation of the membrane; the lower part of the spike (the cap of the mushroom) penetrates deeper into the virus at its circumference than at its centre. It can also be seen (Fig. 4*c*) that the central regions of each face of the icosahedron are connected, just above the lipid membrane, by a pair of high-density bridges crossing the 2-fold axis.

The trimer clustering of the spike proteins bears resemblance to the clustering of influenza virus haemagglutinin¹⁶. Furthermore, the SFV spike protein also contains a hydrophobic sequence thought to initiate the fusion event with the host cell endosome membrane during infection¹⁷. It is tempting to speculate that the fusion sequence is protected by the trimeric clustering. Fusion could then start with the opening of the spike (there is plenty of room for that), exposing the fusion sequence and opening a channel through the spike centre towards the virus membrane at a point where it is made fragile by bending.

During virus maturation, the budding process takes place when the spike proteins, incorporated in the cell membranes, form the envelope around the nucleocapsids coming from the cytoplasm. One possibility is that this process is fully controlled by the nucleocapsid, which would contain the structural information required for defining the position of all the spikes. Alternatively, the spike-spike interaction could define much of the envelope geometry. This second possibility is supported by the persistence of the spike clustering in fused membranes of

Sindbis virus¹⁸. The observations presented here also suggest that there is much interaction between the spikes. In particular, the bonds that the 3-fold spikes seem to form across the 2-fold axes of the icosahedron define a $T=1$ icosahedral shell consisting of 20 spikes. In this geometry, the 60 pentonal spikes could be regarded as filling material. A similar concept has been proposed for tomato bushy stunt virus², although the difference between synthesis taking place in the cytoplasm and the membrane must be kept in mind. A possible pathway for virus budding could then involve the following steps: (1) Spikes in the cell membranes bind weakly in the plane of the membrane. The binding regions of the spikes correspond to the locations of the 2-fold axes in the virus. This cluster formation could be similar to the condensation of a gas into a liquid in two dimensions. The process could be initiated by the trimerization of the spike proteins. (2) The cytoplasmic part of the spike cluster binds to the nucleocapsid. This causes the cell membrane to bend and to lock the bond between the spikes. (3) The process continues with the other spikes, but before a ring of five 3-fold spikes is closed, the central space of the ring is filled with pentonal spikes, which may be only weakly bound. Possibly the virus may not need to have all the pentonal sites occupied. Missing spikes or spike proteins could explain the low values

obtained by direct virus mass determination^{13,14}.

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1. Caspar, D. L. D. & Klug, A. *Cold Spring Harb. Symp. quant. Biol.* **27**, 1-24 (1962).
2. Harrison, S. C. *Adv. Virus Res.* **28**, 175-240 (1983).
3. Harrison, S. C., Olson, A., Schutt, C. E., Winkler, F. K. & Bricogne, G. *Nature* **276**, 368-373 (1978).
4. Rossmann, M. G. *et al. Nature* **317**, 145-153 (1985).
5. Adrian, M., Dubochet, J., Lepault, J. & McDowell, A. *Nature* **308**, 32-36 (1984).
6. Stewart, M. & Vigers, G. *Nature* **319**, 631-636 (1986).
7. Provencher, S. W. & Vogel, R. H. in *Progress in Scientific Computing* Vol. 2 (eds Deuffhard, P. & Hairer, H.) 304-319 (Birkhäuser, Boston, 1983).
8. von Bonsdorff, C.-H. & Harrison, S. C. *J. Virol.* **16**, 141-145 (1975).
9. von Bonsdorff, C.-H. & Simons, K. in *Animal Virus Structure* (eds Nermut, V. & Steven, A. C.) (Elsevier, Amsterdam, in the press).
10. Simons, K. & Garoff, H. *J. gen. Virol.* **50**, 1-21 (1980).
11. Söderlund, H., von Bonsdorff, C.-H. & Ulmanen, I. *J. gen. Virol.* **45**, 15-26 (1979).
12. Lepault, J. & Pitt, T. *EMBO J.* **3**, 101-105 (1984).
13. Freeman, R. & Leonard, K. R. *J. Microsc.* **122**, 275-286 (1981).
14. Simons, K. & Warren, G. *Adv. Protein Chem.* **36**, 79-132 (1984).
15. Rossmann, M. G. *Virology* **134**, 1-11 (1984).
16. Skehel, J. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 968-972 (1982).
17. Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H. & Delius, H. *Nature* **288**, 236-241 (1980).
18. von Bonsdorff, C.-H. & Harrison, S. C. *J. Virol.* **28**, 578-583 (1978).
19. Kam, Z. *J. theor. Biol.* **82**, 15-39 (1980).

Expression of the HTLV-III envelope gene by a recombinant vaccinia virus

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The discovery that the aetiological agent of acquired immune deficiency syndrome (AIDS) is a retrovirus, referred to as human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV) (for review see ref. 1), has raised the possibility of developing a vaccine. In this regard, the envelope (*env*) proteins of murine retroviruses can induce protective immunity in mice^{2,3}. The HTLV-III *env* gene specifies a primary polypeptide of ~860 amino acids that is glycosylated to form a precursor of relative molecular mass (M_r) 160,000 (gp160), which gives rise to mature membrane-associated proteins⁴⁻⁹ of M_r 120,000 (gp120) and 41,000 (gp41). The HTLV-III *env* gene has been expressed in *Escherichia coli*¹⁰ and by simian virus 40 (SV40) vectors⁵ but formation of the authentic proteins has not been demonstrated. Here, we describe the expression of the complete *env* gene by a vaccinia virus vector. Evidence is presented that synthesis, glycosylation, processing and membrane transport of the *env* polypeptide occurred without other HTLV-III gene functions; the *env* protein was recognized by sera from unrelated AIDS patients; and a single vaccination with the infectious recombinant vaccinia virus induced antibodies to gp120 in mice.

DNA segments containing the entire HTLV-III *env* gene starting at, or 70 base pairs (bp) upstream of, the putative translation initiation codon, were placed under the control of a vaccinia virus promoter as described in Fig. 1 legend. Recombinant vaccinia virus plaques were selected on the basis of their thymidine kinase-negative (TK⁻) phenotype and identified by formation of a blue colour, due to expression of β -galactosidase, on addition of an indicator to the agarose overlay¹¹. Synthesis of HTLV-III *env* proteins was demonstrated by polyacrylamide gel electrophoresis of lysates of cells infected with purified recombinant vaccinia viruses v25 and v26. The separated polypeptides were transferred to nitrocellulose and incubated suc-

cessively with serum from an AIDS patient (AIDS serum) and ¹²⁵I-protein A. When H9 cells were used for infection, the three major bands detected by autoradiography co-migrated with authentic gp160, gp120 and gp41 (Fig. 1a); these polypeptides were not detected in lysates of either uninfected cells or cells infected with standard vaccinia virus. Bands corresponding to gp160 and gp41 but not gp120 were detected when HeLa cells (Fig. 1a) or CV-1 cells (not shown) were infected with recombinant vaccinia virus. A minor band migrating with an apparent M_r of ~100,000 (possibly representing a degraded or incompletely glycosylated form of gp120) was noted (Fig. 1a). Formation of gp120 was not specific for H9 lymphocytes, however, as further studies showed that gp120 was also made in NIH 3T3 cells infected with recombinant vaccinia virus.

Glycosylation of the HTLV-III polypeptides synthesized by recombinant vaccinia viruses was demonstrated by metabolically labelling infected H9 cells with ³H-glucosamine. Labelled polypeptides corresponding to gp160, gp120 and gp41 and an additional unidentified glycosylated polypeptide with a M_r of ~80,000, were detected (Fig. 1b).

Pulse-chase experiments provided information regarding the synthesis and processing of the *env* proteins. Autoradiographs of polyacrylamide gels revealed several background bands common to cells infected with both standard and recombinant vaccinia virus (Fig. 2). The band that was labelled most during the pulse-period, however, was specific for the recombinant virus and corresponded in size to gp160. During the chase period, the gp160 band diminished in intensity while bands corresponding to gp120 and gp41 increased, consistent with a precursor-product relationship. Curiously, however, significant amounts of gp41 always formed during the pulse even when the time was reduced to 20 min.

Indirect immunofluorescence demonstrated that surface transport of HTLV-III *env* polypeptides occurs in cells infected with purified recombinant vaccinia virus (Fig. 3). The AIDS serum used for this analysis had been pre-adsorbed with vaccinia virus-infected H9 cells and no discernible immunofluorescence was detected with either uninfected or vaccinia virus-infected H9 cells (data not shown).

Because of sequence variations in the *env* genes of independent virus isolates^{12,13}, the antigenicity of the *env* protein expressed by recombinant vaccinia virus was tested with additional sera. Using an assay similar to that in Fig. 1b, we found that ³H-glucosamine-labelled gp160 and gp120 were specifically immunoprecipitated with sera from six unrelated patients but

not with sera from two normal volunteers. A strong band corresponding to gp41 also was obtained with sera from AIDS patients; however, a faint band in the same position was also observed with normal sera.

To determine immunogenicity, mice were inoculated with purified infectious recombinant vaccinia virus containing the HTLV-III *env* gene or influenza haemagglutinin. Experimental and control sera were then incubated with lysates of HTLV-III-infected H9 cells that had been metabolically labelled with ^{35}S -methionine or ^3H -glucosamine. Induction of antibody was demonstrated by immunoprecipitation of gp120 with sera only from mice that had been inoculated with the HTLV-III *env* gene recombinant. Representative data are shown in Fig. 4.

Recombinant vaccinia viruses have been shown to stimulate humoral^{14,15} and cell-mediated^{16,17} immunity and to protectively immunize experimental animals against a variety of diseases¹⁸⁻²³, including murine retrovirus-induced leukaemia³. Using vaccinia virus as a vector, we have now shown that the HTLV-III *env* proteins can be synthesized, glycosylated,

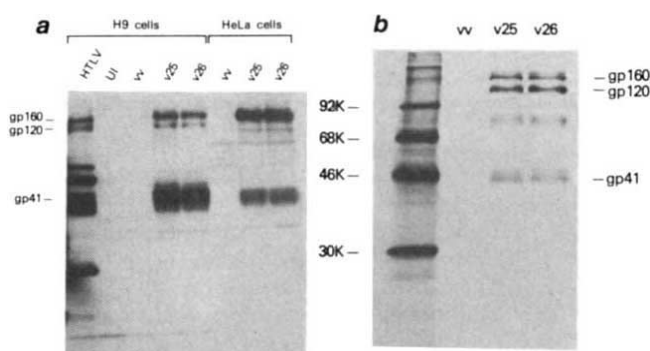


Fig. 1 Expression of HTLV-III *env* gene products by vaccinia virus recombinants. **a**, Immunoblot of HTLV *env* proteins from cells infected with vaccinia virus recombinants. H9 or HeLa cells were infected with 30 plaque-forming units (PFU) of standard vaccinia virus (vv) or vaccinia recombinant virus v25 or v26. Cells were collected 24 h after infection, lysed and subjected to SDS-polyacrylamide (10%) gel electrophoresis. The polypeptides were transferred to a nitrocellulose membrane and incubated successively with serum from a patient with AIDS and ^{125}I -protein A. The positions of authentic *env* proteins gp160, gp120 and gp41 were determined by electrophoresis of HTLV-III-infected H9 cell lysates in a parallel lane. Control uninfected (UI) cells were treated in a similar manner. **b**, Immunoprecipitation of ^3H -glucosamine-labelled HTLV-III *env* proteins. H9 cells were infected with vv, v25 or v26 and metabolically labelled with ^3H -glucosamine from 2 to 24 h. Cell extracts were first incubated with normal donor serum then with protein A-Sepharose to remove proteins with nonspecific binding affinity. After centrifugation, the supernatants were incubated with serum from a patient with AIDS and again with protein A-Sepharose. Proteins were eluted from the Sepharose and resolved on an SDS-polyacrylamide (10%) gel and autoradiographed. The extreme left-hand lane shows marker proteins (M_r represents $M_r = 1,000$).

Methods. A 3.5-kilobase *Sst*I fragment of HTLV-III DNA was excised from λ clone BH8 (ref. 6), blunt-ended with T4 DNA polymerase and cloned into the *Sma*I site of pUC18 so that the *Xba*I and *Pst*I polylinker sites were located upstream of the *env* gene. After cleavage with *Xba*I and *Pst*I, unidirectional digestion with exonuclease III and S_1 (ref. 24) was carried out. Blunt ends were created using the Klenow fragment of DNA polymerase and deoxyribonucleoside triphosphates, and the plasmids were recircularized with T4 DNA ligase and used to transform *E. coli*. Plasmids from individual colonies were sequenced to determine the number of nucleotides remaining upstream of the *env* gene. DNA fragments containing the entire *env* gene were obtained by cleavage with *Sph*I and *Sst*I and inserted into the *Sma*I site of pSC11 (ref. 11) to form pSC25 and pSC26, which have 0 and 70 bp of *env* leader sequence, respectively. Recombinant viruses v25 and v26, which are both TK^- and β -galactosidase-positive, were formed by homologous recombination and plaque-purified.

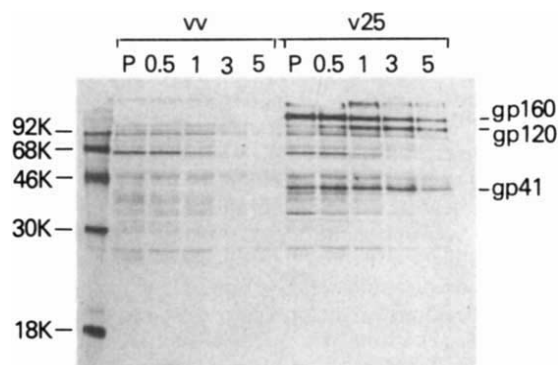


Fig. 2 Synthesis and processing of HTLV-III *env* proteins. At 2 h after infection with wild-type (vv) or recombinant vaccinia virus v25, the H9 cells were incubated for 1 h in methionine-free medium, then pulse-labelled with ^{35}S -methionine for 30 min. The cells were then resuspended in medium containing excess methionine for 0.5, 1, 3 and 5 h, as indicated. Immunoprecipitation and polyacrylamide gel electrophoresis were done as described in Fig. 1 legend. The left-hand lane shows marker proteins.

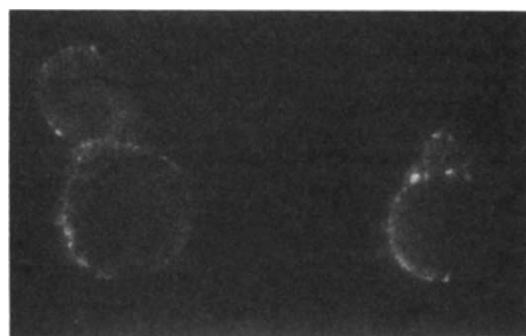


Fig. 3 Detection of HTLV-III *env* protein on the surface of H9 cells infected with recombinant vaccinia virus. H9 cells, infected with 30 PFU of v25 per cell for 12 h, were collected by centrifugation, washed three times with phosphate-buffered saline (PBS), and incubated for 30 min with AIDS patient serum that had been pre-adsorbed on vaccinia virus-infected H9 cells. After washing repeatedly with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG and examined by fluorescence microscopy.

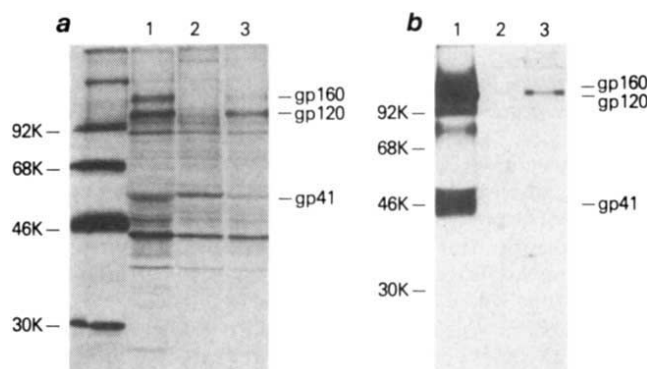


Fig. 4 Induction of antibodies to gp120 by immunization of mice with recombinant vaccinia virus. H9 cells were infected with HTLV-III and labelled with ^{35}S -methionine (**a**) or ^3H -glucosamine (**b**). The cell lysates were incubated with sera from an AIDS patient (lane 1), or from a representative mouse that had been inoculated intraperitoneally with 10^8 PFU of a recombinant vaccinia virus containing the influenza haemagglutinin gene (lane 2) or HTLV-III *env* gene (lane 3). The antibody-bound proteins were immunoprecipitated with protein A-Sepharose and analysed by polyacrylamide gel electrophoresis as described in Fig. 1 legend.

processed and transported to the plasma membrane without expression of other retrovirus genes. Thus, recombinant vaccinia viruses should prove useful for further analysis of the processing of the *env* protein, to prepare native antigens free of other HTLV-III proteins, make target cells for studying cell-mediated immunity, and to obtain polyclonal and monoclonal antibodies to the *env* protein. Further work is needed to determine whether antibody to gp120 is neutralizing and whether vaccination with live recombinant vaccinia virus or with inactivated materials from infected cells might have immunoprophylactic value in preventing the development of AIDS. It is important to note that use of live vaccinia virus in individuals that already have AIDS would be contraindicated because of the suppression of their immune systems.

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1. Wong-Staal, F. & Gallo, R. C. *Nature* **317**, 395-403 (1985).
2. Hunsmann, G., Schneider, J. & Schulz, A. *Virology* **113**, 602-612 (1981).
3. Earl, P. L., Chesebro, B. & Moss, B. in *Vaccines 86* (eds Lerner, R. A., Chanock, R. M. & Brown, F.) (Cold Spring Harbor Laboratory, New York, in the press).
4. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. *Cell* **40**, 9-17 (1985).
5. Sanchez-Pescador, R. *et al. Science* **227**, 484-492 (1985).
6. Ratner, L. *et al. Nature* **313**, 277-284 (1985).
7. Muesing, M. A. *et al. Nature* **313**, 450-458 (1985).
8. Robey, W. G. *et al. Science* **228**, 593-595 (1985).
9. Veronese, F. D. *et al. Science* **229**, 1402-1405 (1985).
10. Crowl, R. *et al. Cell* **41**, 979-986 (1985).
11. Chakrabarti, S., Brechling, K. & Moss, B. *Molec. cell. Biol.* **5**, 3403-3409 (1985).
12. Ratner, L., Gallo, R. C. & Wong-Staal, F. *Nature* **313**, 636-637 (1985).
13. Binn, S. *et al. Science* **230**, 949-951 (1985).
14. Smith, G. L., Mackett, M. & Moss, B. *Nature* **302**, 490-495 (1983).
15. Panicali, D., Davis, S. W., Winberg, R. L. & Paoletti, E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5364-5368 (1983).
16. Binnink, J. R., Yewdell, J. W., Smith, G. L., Moller, C. & Moss, B. *Nature* **311**, 578-579 (1984).
17. Yewdell, J. W., Binnink, J. R., Smith, G. L. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1785-1789 (1985).
18. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 193-197 (1984).
19. Smith, G. L., Murphy, B. R. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7155-7159 (1983).
20. Moss, B., Smith, G. L., Gerin, J. L. & Purcell, R. H. *Nature* **311**, 67-69 (1984).
21. Mackett, M., Yilma, T. Y., Rose, J. A. & Moss, B. *Science* **227**, 433-435 (1985).
22. Cremer, K. J., Mackett, M., Wohlenberg, C., Notkins, A. L. & Moss, B. *Science* **228**, 737-740 (1985).
23. Wiktor, T. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7194-7198 (1984).
24. Henikoff, S. *Gene* **28**, 351-359 (1984).

Expression of AIDS virus envelope gene in recombinant vaccinia viruses

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Acquired immune deficiency syndrome (AIDS) is an infectious disease characterized by severe impairment of the patient's cell-mediated immune system^{1,2}. Several lines of evidence have indicated that the aetiological agent of AIDS is a group of T-lymphotropic retroviruses, variously known as lymphadenopathy-associated virus (LAV)³, human T-lymphotropic virus type III (HTLV-III)^{4,5} and AIDS-associated retrovirus (ARV)⁶. Serological surveys have indicated that as many as one million people in the United States may have been infected by LAV/HTLV-III⁷, and the spread of AIDS has become a global concern. The need for a better understanding of the viral immunology and for a vaccine against AIDS is self-evident. To this end, we have constructed recombinant vaccinia viruses containing the envelope (*env*) gene of LAV, and demonstrate here that cells infected with these viruses express immunoreactive proteins similar to those present on LAV virions. Experimental animals infected with these recombinant viruses elicited antibodies that specifically recognized LAV envelope proteins.

The proviral genome of LAV has been cloned⁸ and its complete nucleotide sequence determined⁹. As deduced from open reading frames in the nucleotide sequence, the genome of LAV contains not only the typical retroviral genes coding for the core proteins (*gag*), the reverse transcriptase (*pol*) and envelope proteins (*env*), but also two novel genes of unknown function, Q and F (ref. 9). We chose to express the *env* gene of LAV as for many viruses it is the major envelope glycoprotein that elicits neutralizing or protective responses.

To insert the LAV *env* gene into vaccinia virus vectors, we used a general procedure described by Mackett *et al.*¹⁰. Briefly, a foreign gene is first inserted into a plasmid vector (pGS20) downstream from a vaccinia transcriptional control element (7.5K promoter). This chimaeric gene in the recombinant plasmid is flanked by vaccinia sequences encoding the viral thymidine kinase gene (*tk*). The plasmid is then introduced into cells previously infected by wild-type vaccinia virus (strain WR); recombination occurs in the *tk* region, which is homologous for both the viral DNA and the plasmid, and allows the insertion of the chimaeric gene into the genome of the vaccinia virus. The resultant recombinant virus will be TK⁻ and will grow in selective medium containing 5-bromodeoxyuridine. By using this approach, we constructed two recombinant vaccinia viruses, v-env5 and v-env2. Recombinant v-env5 contains the entire envelope coding sequence (nucleotides 5,767 to 8,349)⁹ as well as 96 base pairs (bp) of 5'-proximal and 223 bp of 3'-proximal untranslated sequences. Recombinant v-env2 contains the *KpnI* fragment (5,889-8,572) and does not carry the sequence encoding the N-terminal 42 amino acids of *env*. The *KpnI* fragment was inserted into v-env2 using oligonucleotide linkers so that a translational initiation codon was introduced in-phase with the reading frame of the envelope coding sequences (Fig. 1).

To determine whether the LAV *env* gene was expressed by the recombinant vaccinia viruses, we infected two cell lines (BSC-40 and HeLa) with v-env5 and v-env2 and used Western blotting to assay for LAV-specific proteins in the infected cell lysate. Three major proteins immunoreactive with pooled serum from seropositive individuals were detected in the v-env5-infected cells (Fig. 2). The relative molecular masses (*M_r*) of these proteins were estimated to be 150,000 (150K), 120K and 41K, similar to those of LAV envelope glycoproteins gp150, gp110 and gp41 (refs 11, 12 and F. Clavel, personal communication). Radioisotope labelling with glucosamine indicated that these recombinant-made proteins, like the envelope proteins of LAV, were also glycosylated (Fig. 3a, lane 7). Differences in the glycosylation patterns could account for the slight variations observed in the electrophoretic mobilities of recombinant-made proteins compared with LAV virion glycoproteins (data not shown). It has been suggested that gp150 of LAV is the precursor from which an exterior protein gp110 and a transmembrane protein gp41 are derived^{9,11,12}. Our results (Fig. 3b, lanes 7-12) indicated the same precursor-product relationship for the recombinant-made 150K, 120K and 41K proteins. The processing of the 150K protein appeared to be slow and inefficient in HeLa cells, as by 6 h after pulse-labelling less than 50% of the radioactivity in the 150K protein was chased into 120K and 41K proteins. Preliminary results indicated that this processing was more efficient in certain types of human peripheral blood cells infected with the same recombinant virus (data not shown). As would have been expected for gp110 of LAV, the recombinant-made 120K protein was also detected in infected cell medium (Fig. 3c, lane 3). These results demonstrated that the recombinant virus v-env5 is able to express the LAV *env* gene and to produce immunoreactive proteins that are processed in a pattern similar to that of authentic LAV envelope glycoproteins.

Recombinant virus v-env2 lacked the putative signal sequence for LAV *env*, but was able to produce at least three immunoreactive polypeptides: 99K, 68K and 40K (Fig. 2, lane 4). Results from pulse-chase experiments indicated that the *env* sequence in v-env2 was expressed as an unmodified 87K precursor (780

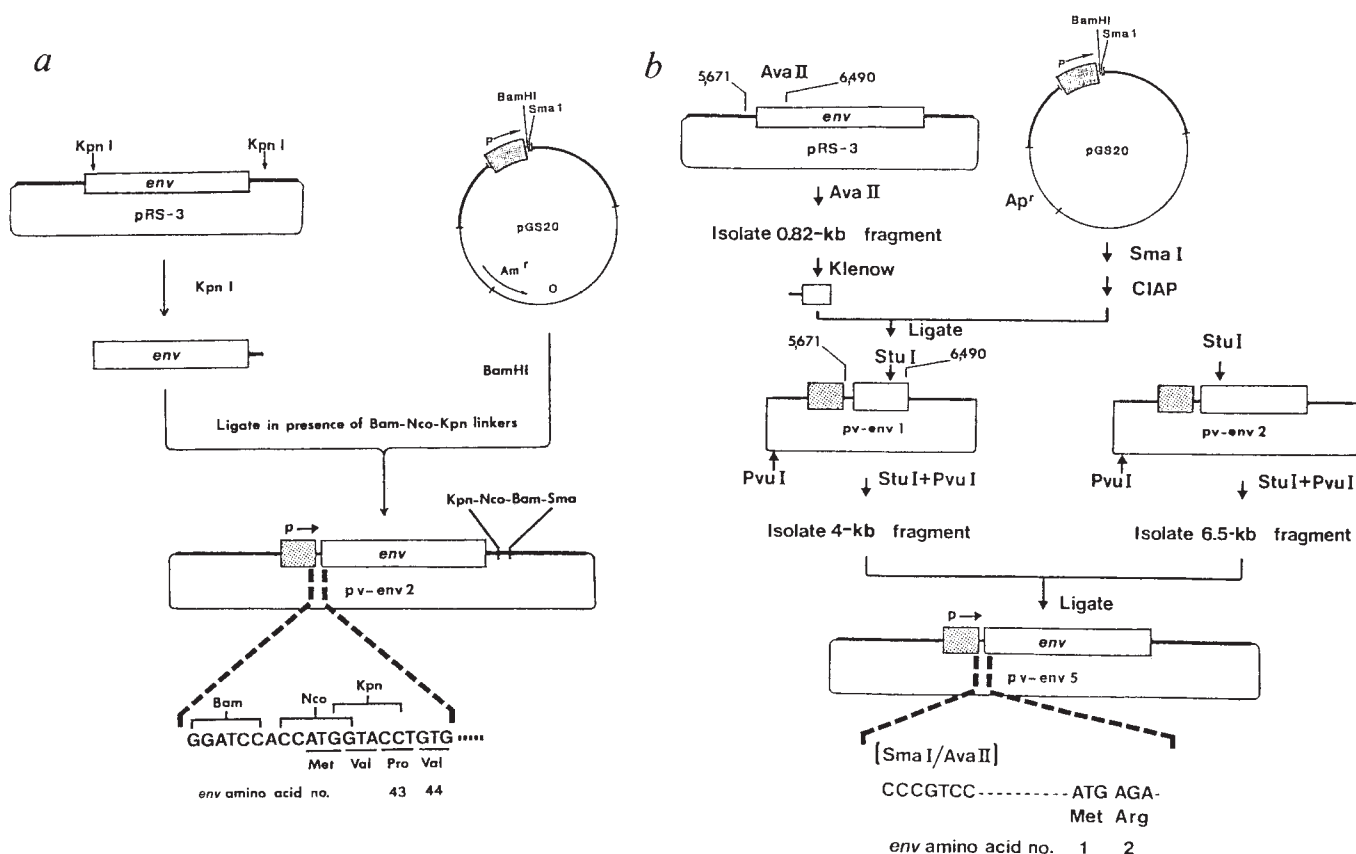


Fig. 1 Construction of plasmid vectors containing vaccinia promoter and LAV envelope coding sequences. **a**, Construction of vaccinia-LAV recombinant plasmid lacking the sequence coding for the first 42 amino acids of *env*. Plasmid *pRS-3*, which contains LAV-specific sequences from the *Eco*RI site at nucleotide 5,289 (ref. 9) to the *Sst*I site at 9,129 inserted at the corresponding sites of plasmid vector *pUC18*, was digested with *Kpn*I and a 2.68-kilobase pair (kb) fragment was isolated. This fragment contained LAV-specific DNA (nucleotides 5,889–8,572) and included sequences encoding envelope proteins from amino acid 42 to the carboxy-terminus. This fragment was ligated with *Bam*HI-digested *pGS20* DNA in the presence of the oligodeoxynucleotide linkers 5'-GATCCACCATGGTAC-3' OH and 5'-CATGGTG-3' OH. These linkers were designed (1) to allow ligation of the *Bam*HI and *Kpn*I cohesive ends of the two different DNA molecules and (2) to provide a translational initiation codon in-phase with the envelope coding sequences. The ligation mixture was used to transform *Escherichia coli* strain MC1000. Plasmid DNA from ampicillin-resistant transformants was tested for the orientation of the insert and the regeneration of *Bam*HI, *Nco*I and *Kpn*I sites at the ligation junction. The nucleotide sequence at the ligation junction of the desired plasmid, *pv-env2*, is as indicated. The vaccinia 7.5K promoter is represented by the shaded area and the direction of transcription by the arrow. **b**, Construction of vaccinia-LAV recombinant plasmid containing the entire envelope coding sequence. This plasmid was constructed in two steps. First, a 0.82-kb *Ava*II fragment from *pRS-3* was purified. This fragment contains LAV-specific sequences (nucleotides 5,671 to 6,490) including those encoding the N-terminal portion of the envelope protein and 95 bp of 5'-untranslated sequences. The fragment was blunt-ended with Klenow enzyme and ligated with plasmid *pGS20* that had been linearized with *Sma*I and dephosphorylated with calf-intestine alkaline phosphatase (CIAP). Recombinant plasmid with the structure indicated was isolated and designated *pv-env1*. The second stage of construction involved rejoining the entire *env* sequence at the *Stu*I site present in both *pv-env1* and *pv-env2*; this was done by ligating the 4-kb *Stu*I and *Pvu*I fragment of *pv-env1*, which contained the vaccinia promoter and the 5' portion of the *env* gene, with the 6.5-kb fragment of *pv-env2* generated by the same restriction enzymes and containing the remaining portion of the *env* gene. The resultant plasmid, *pv-env5*, contained the entire envelope gene as well as 95 bp of 5'-proximal and 223 bp of 3'-proximal adjacent sequences. To insert the chimaeric vaccinia-LAV *env* gene into the vaccinia virus genome, the generalized method described by Mackett *et al.*¹⁰ was used. All recombinant DNA techniques used are described in ref. 21.

Fig. 2 Expression of LAV envelope-related proteins derived from vaccinia-LAV recombinants. Confluent monolayers of BSC-40 or HeLa cells were infected with recombinant virus *v-env5* or *v-env2* at a multiplicity of infection (MOI) of 50 PFU per cell; 12 h after infection the cells were washed twice in phosphate-buffered saline (PBS), resuspended in Laemmli sample buffer and boiled for 4 min. Proteins from infected cells were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred to nitrocellulose membrane, and reacted with pooled serum from LAV/HTLV-III-seropositive individuals. Immunoreactive proteins were detected using protein A, which was labelled with ¹²⁵I by the chloramine-T method. The sources of protein in lanes 1–8 are: 1 and 5, mock-infected cells; 2 and 6, cells infected with wild-type vaccinia virus; 3 and 7, *v-env5*-infected cells; 4 and 8, *v-env2*-infected cells. LAV virion proteins purified on a sucrose gradient were used as a control (LAV) and the positions of envelope proteins gp150, gp110 and gp41 were as indicated. *M_r* standards are shown on the left ($\times 10^{-3}$).

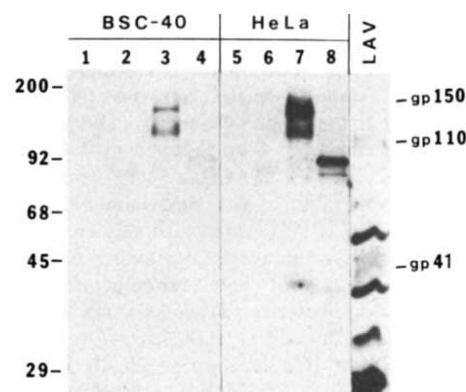


Fig. 3 Radioimmunoprecipitation analysis of LAV envelope-related proteins produced by vaccinia-LAV recombinant viruses. **a**, ^3H -glucosamine labelling of recombinant-produced proteins. HeLa cells were either mock-infected (lanes 1, 5) or infected separately with wild-type vaccinia virus (lanes 2, 6), recombinant v-env5 (lanes 3, 7) or v-env2 (lanes 4, 8), all at a MOI of 50 PFU per cell. ^3H -glucosamine ($0.25 \mu\text{Ci}$, 23 mCi mg^{-1} ; Amersham) was added to culture medium from 4 to 16 h after infection. Cells were washed twice with PBS and lysed in buffer containing 0.1 M NaCl, 0.01 M Tris pH 7.4, 1 mM EDTA, 1% Nonidet P-40 (NP40) and 0.5% sodium deoxycholate. Aliquots of cell lysates were mixed with either normal human serum (lanes 1-4) or pooled serum from LAV/HTLV-III-seropositive individuals (lanes 5-8). Immunoreactive proteins were precipitated by fixed *Staphylococcus aureus* cells bearing protein A, resolved by SDS-PAGE and detected by fluorography. **b**, 'Pulse-chase' analysis of LAV envelope-related proteins produced by vaccinia-LAV recombinants. Confluent monolayers of HeLa cells were infected with wild-type vaccinia virus (lanes 1-6), recombinant v-env5 (lanes 7-12) or v-env2 (lanes 13-18), all at a MOI of 50 PFU per cell. At 10.5 h post-infection, cells were labelled with ^{35}S -methionine ($>800 \text{ Ci mmol}^{-1}$; Amersham) at $100 \mu\text{Ci ml}^{-1}$ for 15 min. At the end of the labelling period, cells were washed once with 2 ml of pre-warmed chase medium (Dulbecco's modified Eagle's medium, 3 mg ml^{-1} L-methionine, 5% calf serum, 100 U ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin) and re-fed with 1 ml of the same medium before being returned to the incubator. At various times afterwards, cells were washed and lysed as described for **a** and proteins from cell lysates were immunoprecipitated with pooled serum from LAV/HTLV-III-seropositive individuals. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by fluorography. The duration of each chase was as follows (h): 0 (lanes 1, 7, 13); 0.5 (lanes 2, 8, 14); 1 (lanes 3, 9, 15); 2 (lanes 4, 10, 16); 6 (lanes 5, 11, 17) and 12 (lanes 6, 12, 18). **c**, Presence of LAV envelope-related protein in the growth medium of cells infected with vaccinia-LAV recombinant viruses. Confluent monolayers of HeLa cells were either mock-infected (lane 1), or infected separately by wild-type vaccinia virus (lane 2), recombinant v-env5 (lane 3) or v-env2 (lane 4), all at a MOI of 20 PFU per cell. Cells were labelled with ^{35}S -methionine ($>800 \text{ Ci mmol}^{-1}$; Amersham) at $100 \mu\text{Ci ml}^{-1}$ from 10 to 12 h after infection. At the end of the labelling period, medium was removed and clarified by centrifugation for 2 min at $12,000g$ before immunoprecipitation using pooled serum from LAV/HTLV-III-seropositive individuals. Proteins from infected cells immunoprecipitated by the same serum are shown in the panel labelled P (for pellet) and those from the medium in the panel labelled S (for supernatant).

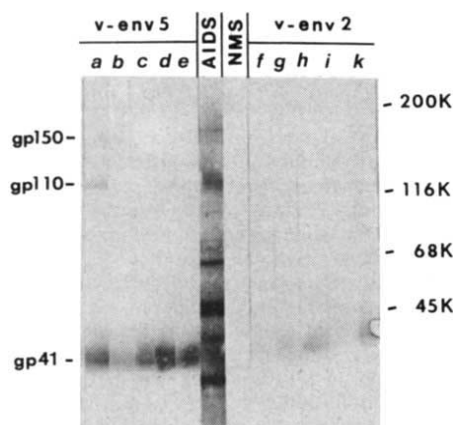
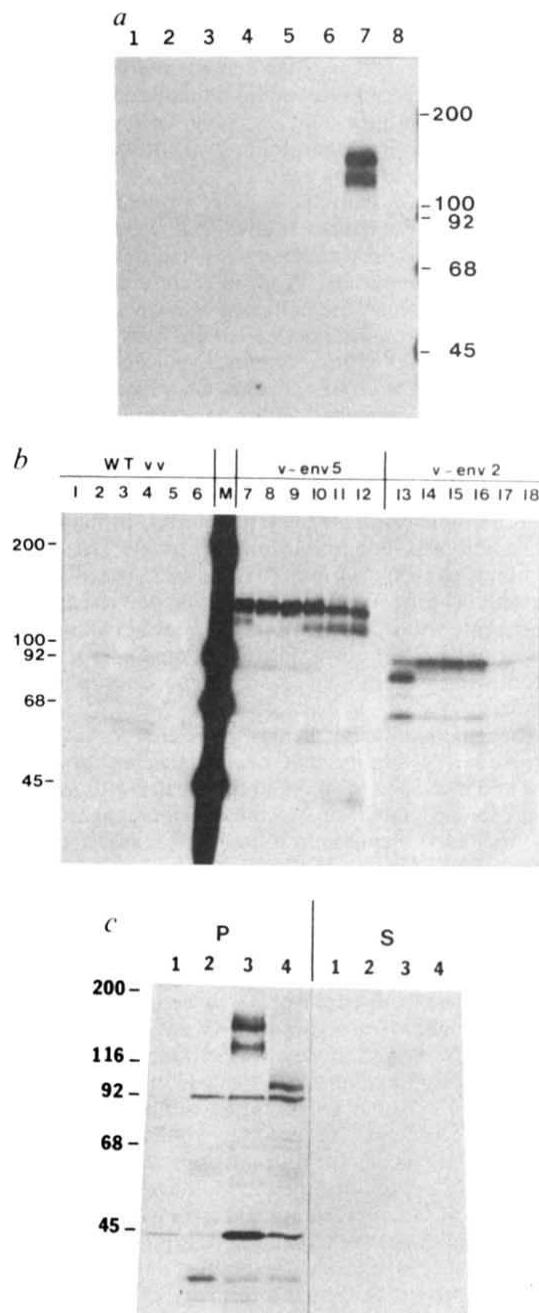


Fig. 4 Western blot analysis of serum samples from mice immunized with vaccinia-LAV recombinant viruses. **a-e**, Serum samples from five mice inoculated with v-env5; **f-k**, samples from individuals inoculated with v-env2. Pooled sera from LAV/HTLV-III-seropositive individuals (AIDS) and non-immunized C57BL/6J mice (NMS) were used as positive and negative controls, respectively. The positions of the LAV envelope glycoproteins gp150, gp110 and gp41 are indicated.

Methods. Male inbred mice (C57BL/6J, Jackson Laboratory) at 5-7 weeks old were immunized by tail scarification, in which a $10\text{-}\mu\text{l}$ inoculum containing 2×10^7 PFU of recombinant vaccinia virus was applied to abrasions generated with a bifurcated needle at the base of the tail. Animals were bled from the retro-orbital sinus at 8 weeks post-inoculation and the serum kept frozen until use. Aliquots of serum samples diluted 50-fold in PBS plus 0.2% NP40 plus 3% non-fat dry milk were reacted with LAV virion proteins resolved by SDS-PAGE and immobilized on nitrocellulose filters by electro-transfer. LAV proteins recognized by these sera were detected using goat anti-mouse immunoglobulin conjugated with alkaline phosphatase.

amino acids), which was processed to a 99K intermediate and cleaved into the 68K and 40K polypeptides (Fig. 3b, lanes 13–18). As would have been expected for proteins lacking signal peptides, no LAV envelope-related polypeptide was detected in the infected cell medium (Fig. 3c, lane 4) and no N-linked glycosylation with ^3H -glucosamine was observed (Fig. 3a, lane 8).

We examined the immunogenicity of proteins expressed by recombinant vaccinia viruses v-env5 and v-env2. Male mice (strain C57BL/6J) were inoculated by tail scarification with 2×10^7 plaque-forming units (PFU) of each of the recombinant viruses. Serum samples were collected at 2-week intervals for 8 weeks post-inoculation and were analysed by Western blotting for reactivity with LAV virion proteins. By 8 weeks post-inoculation, all animals immunized with the recombinant viruses produced antibodies that reacted with LAV envelope glycoprotein gp41 (Fig. 4). Serum from some of the animals immunized with v-env5 (Fig. 4a and our unpublished results) also recognized gp150 and gp110, indicating the ability of this recombinant virus to elicit an immune response to all major glycoproteins of LAV. Using enzyme-linked immunosorbent assay (ELISA) and Western blot analyses, we found that the LAV-specific antibody titres elicited by both recombinants continued to rise throughout the experiment, and that recombinant v-env5 was also able to elicit LAV-specific responses in an out-bred strain of mouse (ICR) following several routes of immunization (data not shown).

The description of neutralizing antibodies to LAV/HTLV-III¹³ raises the possibility that neutralizing epitopes can be identified and a vaccine formulated for the prevention of AIDS. One promising approach for vaccine development is the use of vaccinia virus as an expression vector^{14,15}. A vast database has been established detailing the safety and efficacy of vaccinia virus as a successful smallpox vaccine, and there is an increasing body of knowledge concerning the use of recombinant vaccinia viruses to stimulate humoral^{16–19} and cell-mediated²⁰ immunity against various pathogens. The vaccinia-LAV recombinants that we have described here could prove to be useful tools for the study of the antigenic properties of LAV envelope proteins and eventually for the design of an effective vaccine against AIDS.

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Note added in proof: The immunogenicity of v-env5 has been examined in one species of sub-human primate (*Macaca fascicularis*). Seven out of 8 vaccinated animals developed specific antibodies to the envelope glycoprotein of LAV.

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- Gottlieb, M. *et al.* *New Engl. J. Med.* **305**, 1426–1431 (1981).
- Masur, H. *et al.* *New Engl. J. Med.* **305**, 1431–1438 (1981).
- Barré-Sinoussi, F. *et al.* *Science* **220**, 868–870 (1983).
- Gallo, R. C. *et al.* *Science* **224**, 500–502 (1984).
- Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. *Science* **224**, 497–499 (1984).
- Levy, J. A. *et al.* *Science* **225**, 840 (1984).
- Curran, J. W. *et al.* *Science* **229**, 1352–1357 (1985).
- Alizon, M. *et al.* *Nature* **312**, 757–760 (1985).
- Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. *Cell* **40**, 9–17 (1985).
- Mackett, M., Smith, G. L. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **81**, 857–864 (1984).
- Montagnier, L. *et al.* *Virology* **144**, 283–289 (1985).
- Robey, W. G. *et al.* *Science* **228**, 593–595 (1985).
- Robert-Guroff, M., Brown, M. & Gallo, R. C. *Nature* **316**, 72–74 (1985).
- Panicali, D. & Paoletti, E. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4927–4931 (1982).
- Mackett, M., Smith, G. L. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7415–7419 (1982).
- Smith, G. L., Mackett, M. & Moss, B. *Nature* **302**, 490–495 (1983).
- Panicali, D., Davis, S. W., Weinberg, R. L. & Paoletti, E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5364–5368 (1983).
- Smith, G. L., Murphy, B. R. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7155–7159 (1983).
- Paoletti, E. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 193–197 (1984).
- Bennick, J. R. *et al.* *Nature* **311**, 578–579 (1984).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (eds) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).

Requirement for c-ras proteins during viral oncogene transformation

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Many retroviral oncogenes have been classified into one of several categories based on structure, enzymology and cellular localization¹. These genes originated from host cells and are probably derived from genes normally involved in the control of cell proliferation². The cellular counterparts of three oncogenes have been identified as a growth factor or growth factor receptor^{3–6}; related oncogenes include receptor-like membrane proteins which often express tyrosine kinase activity. These growth factor-related oncogenes are structurally and biochemically distinct from the membrane-associated *ras* gene family, which bind and hydrolyse GTP^{7–9}. Oncogenes localized primarily in the cytoplasm which probably have serine kinase activity, have also been identified^{10–12}. Although the structure and biochemistry of many oncogenes have been extensively studied, relatively little is known about the functional relationships of oncogene proteins within the cell. An opportunity to study such interaction is provided by the identification of a monoclonal antibody that neutralizes cellular *ras* proteins when microinjected into cells¹³. It has been shown previously that the injected antibody inhibits the initiation of S-phase in NIH 3T3 cells¹⁴. In the present study we injected this monoclonal antibody into NIH 3T3 cells transformed by a variety of oncogenes. The results show that transformation by three growth factor receptor-like oncogenes depends on c-ras proteins, while transformation by two cytoplasmic oncogenes appears to be independent of c-ras protein.

Anti-ras monoclonal antibodies originally prepared by Furth *et al.*¹⁵ have been analysed extensively. Monoclonal antibody Y13-259 (which binds the c-ras proteins of a variety of species¹⁵) neutralized the activity of co-injected, purified *ras* protein, and

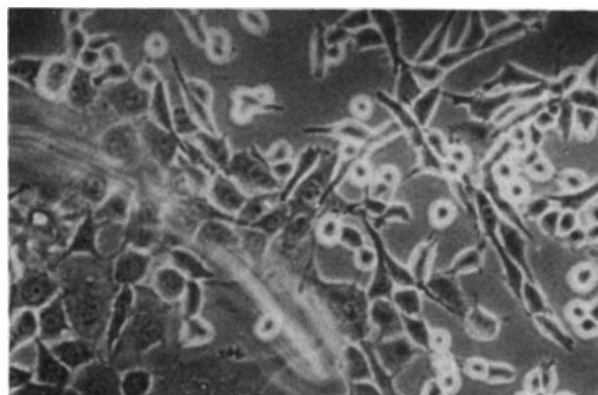


Fig. 1 Morphological reversion of *src*-transformed cells following injection of anti-ras antibody. These NIH 3T3 cells were transformed by the Rous sarcoma viral genome (see Table 1). Neutralizing anti-ras antibody 259 was injected into the cells adjacent to or within a circular mark on the underside of the coverslip to which the cells were attached. A small section of this circular mark is visible, allowing identification of the injected area in the lower left half of this phase-contrast photomicrograph ($\times 82$). After 16 h, cells in the injected area had reverted to the flattened, non-refractile appearance of untransformed cells. The nuclei of most injected cells, for example, are clearly visible. Uninjected cells (or cells injected with control antibody 238; data not shown) remained refractile, with the spherical or spindle shape of transformed cells.

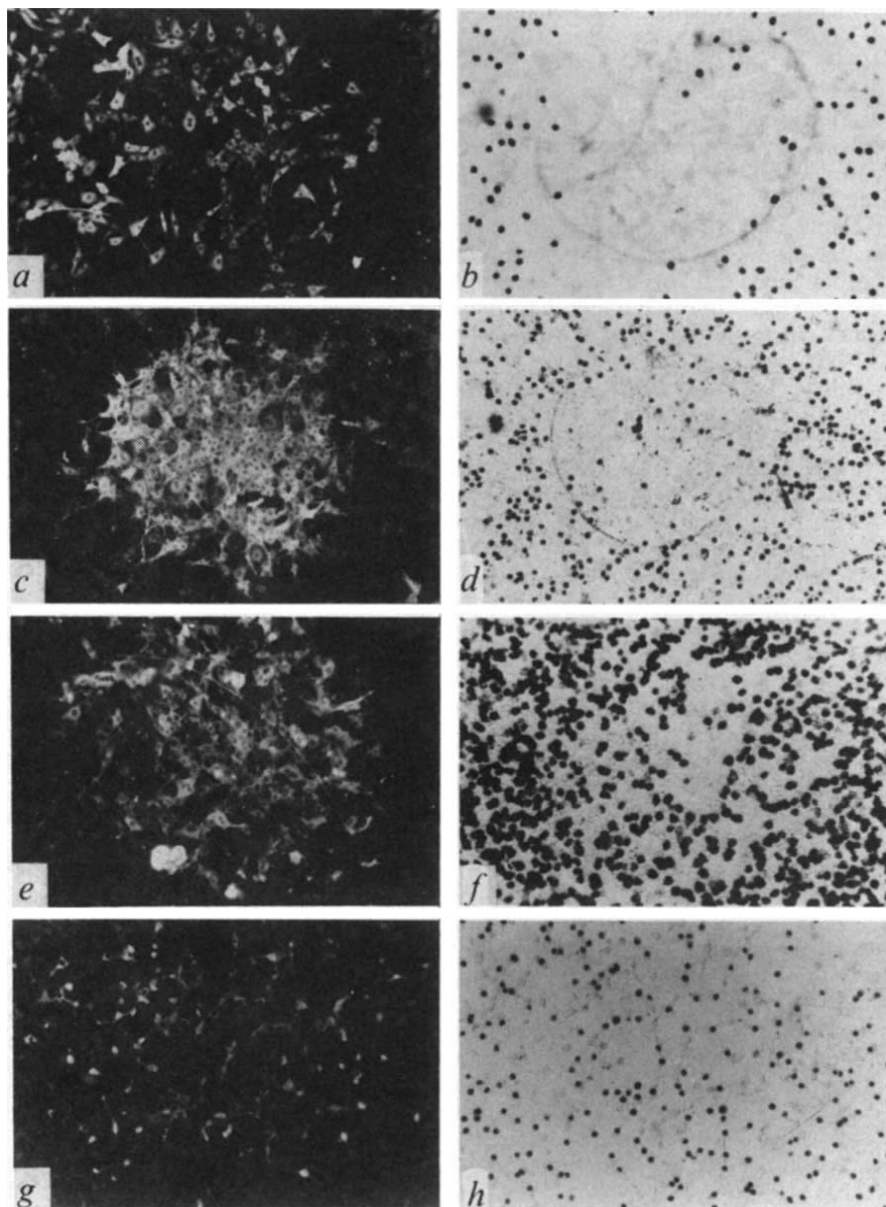


Fig. 2 ^3H -thymidine incorporation into NIH 3T3-transformed cells after antibody injection. NIH 3T3 cells transformed by the *fes* (a, b) or *fms* (c, d) oncogenes (see Fig. 1), the bovine papillomavirus (e, f; from D. R. Lowy²⁵), or the *mos* oncogene (g, h) were grown as a uniform monolayer. Cells within a small circle marked on the back of the coverslips were injected with antibody 259. After 18 h the cells were pulsed with ^3H -thymidine and stained with a fluorescent antibody that recognizes injected antibody within the cells¹⁶. Fluorescence photomicrographs (left) indicate that almost all the cells in the centre of the photographs were successfully injected. Few *fms* or *fes*-transformed cells incorporated thymidine after injection, while a definite reduction with BPV-transformed cells was observed. No reduction in thymidine incorporation was observed in *mos*-transformed cells.

induced a morphological reversion to the normal phenotype in *ras*-transformed NIH 3T3 cells. Antibody Y13-238 neither neutralized co-injected protein nor affected *ras*-transformed cells¹³ and was used as a negative control. When neutralizing antibody 259 was microinjected into untransformed NIH 3T3 cells, their rate of proliferation was decreased by approximately 90% compared with either uninjected cells or cells injected with control antibody 238. The antibody inhibited only the initiation of a new round of DNA synthesis. Inhibition was observed until just before the initiation of S-phase but did not affect a cycle of DNA synthesis once it had begun¹⁴. Cell lines tested in this study were therefore pulsed for 3 h with labelled thymidine, beginning 15–21 h after antibody injection to ensure that any cycle of DNA synthesis in progress at the time of injection would have ended prior to labelling of the cells. Autoradiography would, therefore, reveal thymidine uptake only in cells able to initiate DNA synthesis following antibody injection.

To test the involvement of c-*ras* proteins in transformation, antibody was injected into NIH 3T3 cells transformed by several oncogenes. An attempt was made to inject all transformed cells within a circular area marked on a coverslip; fluorescent antibodies that could recognize the injected immunoglobulin within the cells were used at the end of each experiment to determine

which cells had been successfully injected¹⁶. Fluorescence staining also ensured that the injected antibody remained within the cell for the duration of the experiment. The proportion of injected cells able to incorporate thymidine was determined by comparing the results of fluorescent staining with those of autoradiography; this number was divided by the proportion of uninjected cells in S-phase on the same coverslip to give values for labelling efficiency¹⁶. The latter values indicate the percentage of injected cells which incorporated thymidine compared with the number expected to have been labelled if no injection had occurred. In all cells injected with control antibody 238 the labelling efficiency was between 85 and 115%, with average values near 100%; this was the case with each cell line tested here (data not shown) as well as with numerous cell lines tested in other studies^{14,16}. A labelling efficiency close to 100% indicates that injected and uninjected cells enter S-phase with equal efficiency after injection of control antibody.

NIH 3T3 cells were transformed with membrane-associated, receptor-like proteins (*src*, *fms* and *fes*^{2,17–20}) by transfection of cloned viral DNA. Approximately 12–15 h after microinjection of antibody 259, a distinct morphological reversion from the transformed phenotype was observed in cells that had been transformed by each of the three viral oncogenes. Transformed

cells that had a rounded or spindle-shaped morphology with a refractile appearance reverted to the flattened shape of untransformed NIH 3T3 cells. Figure 1 shows such reversion after injection into *src*-transformed cells. Morphological reversion continued until 30–50 h after injection (at which time injected antibody had disappeared from the cells); then the cells became morphologically transformed once again (data not shown)¹³. In addition to its morphological effects, antibody 259 decreased dramatically the thymidine incorporation of transformed cells labelled during a 3-h pulse between 15 and 24 h after antibody injection. In Fig. 2*a–d* the decrease in thymidine incorporation following injection of antibody into *fms*- and *fes*-transformed cells is clearly visible. Several different clones transformed by each oncogene were analysed. In each of the three transformants labelling efficiencies varied from 10 to 30% (Table 1*a*). For comparison, *ras*-transformed NIH 3T3 cells and untransformed NIH 3T3 cells had labelling efficiencies of 10–15% (Table 1). It is unknown whether the small difference in labelling efficiency between NIH 3T3 cells transformed by *ras* and those transformed by other oncogenes is significant.

Next, we analysed two representatives of the cytoplasmic class of retroviral oncogenes (*mos* and *raf*^{2,21–23}). NIH 3T3 clones transformed by *mos* (from G. F. Vande Woude, R. B. Arlinghaus and R. H. Bassin) and cells infected with *raf*-containing murine sarcoma virus 3611 (aided by two separate helper viruses; prepared by U. R. Rapp) were injected with antibody as before. In no instance of repeated analysis was there any evidence that anti-*ras* antibody 259 altered the transformed morphology of these cells (data not shown). In addition, labelling efficiencies of *mos*- and *raf*-transformed cells were between 90 and 96% (Table 1*a*); this indicates that proliferation was inhibited little, if at all, by injected antibody, as shown in Fig. 2*g, h*, where a direct comparison can be made with cells transformed by other oncogenes. The average labelling efficiency obtained with control antibody 238 was consistently within a few per cent of 100% (data not shown^{14,16}). It is unknown how these oncogenes overcome the normal requirement for *c-ras* in cellular proliferation but it is clear that both representatives of this oncogene class are able to do so. The group of oncogenes that we have shown to depend on *c-ras* proteins during transformation is similar, but not identical, to those reported to be suppressed following fusion to flat revertants of viral *ras*-transformed cells²⁴. Once more is understood about the molecular basis of the morphological reversion, we may find that the differences between the two systems are important.

In addition to retroviruses, several DNA viruses are able to transform cells. The transforming genes of these viruses are not closely related to the sequences of cellular genes or retroviral oncogenes but they may function similarly. These viruses commonly possess multiple transforming genes¹. Transformation by these viruses displays differences in dependence on *c-ras*, just as observed with retroviral oncogenes. NIH 3T3 cells transformed by one such virus, bovine papillomavirus (BPV; from D. R. Lowy²⁵), were tested as described above, together with WI38 and BALB 3T3 cells transformed by simian virus 40 (SV40). Antibody 259 clearly induced morphological reversion in BPV-transformed NIH 3T3 cells (Fig. 2*e, f*) and reduced thymidine incorporation. While the antibody clearly inhibited entry into S-phase in cells transformed by BPV, the labelling efficiencies were normally between 26 and 45% (Table 1*b*). It is unknown whether the difference in labelling efficiencies between cells transformed by this virus and those transformed by the membrane-associated oncogenes is significant. In cells transformed by SV40, on the other hand, the anti-*ras* antibody inhibited proliferation little if at all. While both cell types tested are efficiently inhibited by the antibody prior to transformation¹⁶, labelling efficiencies after SV40 transformation were 86 and 93%, similar to those observed with the *mos* and *raf* oncogenes. It may not be possible at present to make a thorough comparison between the oncogenes of DNA viruses and the

Table 1 Labelling efficiencies of NIH 3T3 cells transformed by various oncogenes

Oncogene	Cell line	Labelling efficiency Average ($\pm$ s.e.m.) (%)	No.†	Source
<i>a</i>	<i>fes</i>			
	NIH 3T3- <i>fes</i> *	10 (2.3)	9	C. J. Sherr ¹⁹
	NIH 3T3- <i>fes</i>	30 (3.9)	10	R. H. Bassin
	<i>fms</i>			
	NIH 3T3- <i>fms</i> *	17 (3.0)	5	C. J. Sherr ¹⁸
	NIH 3T3- <i>fms</i>	21 (4.9)	7	R. H. Bassin
	<i>mos</i>			
	MSV-124	90 (10.0)	5	R. B. Arlinghaus
	pHT1v- <i>mos</i>	96 (2.4)	11	D. G. Blair
	NIH 3T3- <i>mos</i>	93 (2.5)	7	R. H. Bassin
<i>b</i>	<i>raf</i>			
	NIHF4-3611-LeuK	93 (4.0)	10	U. R. Rapp
	NIHF4-3611-4070	91 (2.3)	6	U. R. Rapp
	<i>ras</i>			
	HA-8-21	10 (1.8)	4	D. R. Lowy
	HA-8	12 (8.0)	2	D. R. Lowy
	RS-485	13 (1.5)	5	E. H. Chang
	<i>src</i>			
	NIH 3T3- <i>src</i> *	27 (4.1)	3	Ref. 29
	NIH 3T3- <i>src</i>	23 (3.0)	11	R. H. Bassin
<i>b</i>	SV40			
	SVT2‡	93 (5.7)	5	ATCC
	VA-13‡	86 (3.3)	8	ATCC
	BPV			
	S-2	27 (7.7)	4	D. R. Lowy
	ID-14	26 (3.0)	4	D. R. Lowy
	NIH 3T3	10 (1.3)	7	E. P. Reddy
	Swiss NIH 3T3	15 (3.0)	2	ATCC
<i>b</i>	NIH 3T3	14 (1.3)	7	R. H. Bassin

Antibody 259 was microinjected into the cytoplasm of the cell types listed. Each cell type was derived from a cell clone. A 3-h pulse of ³H-thymidine between 18 and 24 h after injection was followed by fixation in methanol and staining with fluorescent antibody to identify injected cells¹⁶. The cell lines listed above were also injected with non-neutralizing antibody 238. For antibody 238 injections, average labelling efficiencies were in the range 95–105% (see text). NIH 3T3 cells transformed with the *sis* oncogene²⁰ showed a labelling efficiency of 17 $\pm$ 2%. In transfected cells not characterized previously, a cloned cell population was analysed by Southern blotting to ensure that the viral oncogene had become incorporated into cellular DNA. Growth in soft agar and morphological examination verified that the transfected cells displayed the phenotypic characteristics of transformed cells. Labelling efficiencies were obtained by first determining the percentage of injected cells which had incorporated thymidine, then dividing this percentage by the percentage of uninjected cells on the same plate which were labelled.

* These cells were transformed by transfection²⁹ of the viral oncogene described in the reference cited. Clones of transformed NIH 3T3 cells obtained in soft agar were tested by Southern analysis to ensure that the transfected oncogene had been incorporated into the cellular genome. At least three separate cell clones were analysed for each oncogene.

† Number of determinations, each of which averaged between 150 and 200 injected cells.

‡ These lines, obtained from the American Type Culture Collection, are SV40-transformed WI38 (VA-13) or BALB 3T3 (SVT2) cells. Both of these cell lines have labelling efficiencies close to 10% prior to transformation (ref. 16 and our unpublished data).

retroviral oncogenes tested here¹, but these data indicate some similarities in their action.

Any conclusion drawn from the present study depends on the specificity of the cellular proteins neutralized by antibody 259. As described above, this antibody neutralizes *ras* protein within the injected NIH 3T3 cell¹³. However, it is possible that another cellular protein is also neutralized by the antibody. To ascertain that it is in fact *c-ras* proteins which are neutralized and responsible for the results described, we tested numerous tumour cell lines. In these tumour lines the phenotype of antibody inhibition strongly correlates with the presence of a mutant *ras* oncogene¹⁶. Furthermore, deletion mutants of the *ras* gene which are biologi-

cally active but which do not bind antibody 259 have been prepared recently; cells transformed by such mutants were not altered morphologically, nor did they show greatly inhibited thymidine incorporation, after injection of antibody 259²⁶.

It is assumed that while viral oncogenes function without normal control, their mechanism of action is similar to that of related cellular genes. Here we have presented evidence to support this idea in the case of growth factor receptor-like molecules and related oncogenes. It is therefore possible that these data might aid in understanding the way in which cellular genes interact in the control of normal proliferation. Our results indicate that some receptor-like oncogenes depend on *ras* proteins while some cytoplasmic oncogenes do not. There are, of course, numerous oncogenes which we have not yet tested which might behave differently from those described here. With this limitation in mind and on the basis of our present data, we propose that an important class of proliferative signals are received at the cell surface by receptor molecules such as growth factor receptors, and the *c-ras* proteins are essential in the transfer of these signals to cytoplasmic effectors having serine kinase activity; the effectors then modify target molecules which are directly involved in initiating a proliferative cycle. Accordingly, if the cytoplasmic effector were mutated such that it functioned without activation, proliferation would continue independently of *c-ras* proteins. Receptor molecules, on the other hand, would always require *c-ras* to stimulate proliferation.

While our data are consistent with the above scheme, they do not exclude many other possibilities involving multiple metabolic pathways and more complex interactions. For example, we have not reported results with nuclear oncogenes owing to their difficulty in transforming NIH 3T3 cells. The proposed scheme is primarily attractive because of its similarity to the carefully studied mechanism of signal transduction involving cyclic AMP. While it is unlikely that cyclic AMP itself regulates proliferation²⁷, G-regulatory proteins with enzymatic similarities to *c-ras* proteins are involved. These regulatory proteins control signal transduction from cell-surface receptors to cytoplasmic serine kinase effector molecules by regulating adenyl cyclase activity²⁸.

While the present study has examined only one aspect of what is likely to be a highly complex system for regulating proliferation, it does provide a means of functionally comparing separate viral oncogenes. Injection of antibody has been used in other studies to characterize the types of molecules responsible for tumour cell proliferation. Like NIH 3T3 cells transformed by *mos* or *raf* genes, many tumour cells show no inhibition of proliferation when injected with anti-*ras* antibody. In this way their proliferation is distinct from that of the normal cell types studied, each of which was efficiently inhibited by the injected antibody¹⁶.

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1. Bishop, J. M. *Cell* **42**, 23–38 (1985).
2. Bishop, J. M. & Varmus, H. E. in *RNA Tumor Viruses: Molecular Biology of Tumor Viruses* 2nd edn (eds Weiss, R., Teich, M., Varmus, H. & Coffin, J.) 999–1108 (Cold Spring Harbor Laboratory, New York, 1984).
3. Doolittle, F. R. *et al. Science* **221**, 275–277 (1983).
4. Downward, J. *et al. Nature* **307**, 521–527 (1984).
5. Waterfield, M. D. *et al. Nature* **304**, 35–39 (1983).
6. Sherr, C. J. *et al. Cell* **41**, 665–676 (1985).
7. Ellis, R. W. *et al. Nature* **292**, 506–511 (1981).
8. Sweet, R. W. *et al. Nature* **311**, 273–275 (1984).
9. McGrath, J. P., Capon, D. J., Goeddel, D. V. & Levinson, A. D. *Nature* **310**, 644–649 (1984).
10. Klotzer, W. S., Maxwell, S. A. & Arlinghaus, R. B. *Virology* **138**, 143–155 (1984).
11. Moelling, K., Heimann, B., Beimling, P., Rapp, U. R. & Sander, T. *Nature* **312**, 558–561 (1984).
12. Papkoff, J., Nigg, E. A. & Hunter, T. *Cell* **33**, 161–172 (1983).

13. Kung, H.-F., Smith, M. R., Bekesi, E., Manne, V. & Stacey, D. W. *Expl. Cell Res.* **162**, 363–371 (1986).
14. Mulcahy, L. S., Smith, M. R. & Stacey, D. W. *Nature* **313**, 241–243 (1985).
15. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43**, 294–304 (1982).
16. Stacey, D. W., DeGudicibus, S. J. & Smith, M. R. (in preparation).
17. Brugge, J. S. & Erikson, R. L. *Nature* **269**, 346–348 (1977).
18. Fedele, L. A., Even, J., Garon, C. F., Donner, L. & Sherr, C. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4036–4040 (1981).
19. Donner, L., Fedele, L. A., Garon, C. F., Anderson, S. J. & Sherr, C. J. *J. Virol.* **41**, 489–500 (1982).
20. Robbins, K. C., Devare, S. G., Reddy, E. P. & Aaronson, S. A. *Science* **218**, 1131–1133 (1982).
21. Blair, D. G., McClements, W. L., Oskarsson, M. K., Fischinger, P. J. & Vande Woude, G. F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3504–3508 (1980).
22. Rapp, U. R. & Todaro, G. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 624–628 (1980).
23. Horn, J. R., Wood, T. G., Murphy, E. C., Blair, D. G. & Arlinghaus, R. B. *Cell* **25**, 37–46 (1981).
24. Noda, M., Selinger, Z., Scolnick, E. M. & Bassin, R. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5602–5606 (1983).
25. Schiller, J. T., Vass, W. C. & Lowy, D. R. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7880–7884 (1984).
26. Papageorge, A. G. *et al. Molec. cell. Biol.* (in the press).
27. Beckner, S. K., Hattori, S. & Shih, T. Y. *Nature* **317**, 71–72 (1985).
28. Gilman, A. *Cell* **36**, 577–579 (1984).
29. Wigler, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 1373–1376 (1979).

Viral particles induce Ia antigen expression on astrocytes

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Recent studies have shown that γ -interferon (IFN- γ) induces the expression of Ia antigen on astrocytes^{1,2}. This observation is of immunological significance because such activated astrocytes can act as antigen-presenting cells, as demonstrated with myelin basic protein for antigen-specific encephalitogenic T-cell lines³. However, the lack of lymphatic drainage in brain and the presence of the so-called blood-brain barrier restricting traffic of cells and macromolecules suggests that IFN- γ may not be readily available, at least during the initial phases of viral infections. The question therefore arises as to whether astrocytes can be induced to express Ia antigens by other signals directly related to viral infection and possibly independent of IFN- γ . In the present report we demonstrate that a neurotropic murine hepatitis virus induces expression of Ia antigen on astrocytes in tissue culture without infection, rendering these brain cells competent to participate directly in the immune response to a viral infection.

The murine coronaviruses are a group of agents causing acute, subacute or chronic infections in mice or rats accompanied by different disease processes⁴. The JHM strain of this group is neurotropic and has been shown to induce acute or subacute encephalomyelitides which depend on virus as well as host factors⁵. One important factor in the case of subacute encephalomyelitis in Lewis rats is the immune response, which is directed not only against the virus but also against brain tissue⁶. As various types of central nervous system (CNS) disease are associated with this neurotropic murine coronavirus strain, we chose this virus to define its interaction with rat brain cells in culture with respect to the induction of Ia antigen on astrocytes.

As a baseline for our study, we analysed the response of Lewis primary glial cell cultures consisting of macrophages carrying Fc receptors (Fc receptor⁺) and astrocytes expressing glial fibrillary acidic protein (GFAP⁺) (Fig. 2a–c) to recombinant rat IFN- γ (10 U ml⁻¹; Fig. 1a). Recombinant rat IFN- γ induced the expression of Ia on numerous cells in the primary cultures. Fluorescence-activated cell sorting showed that 3–10% of the cells were induced to express Ia compared with the control after 18 h treatment with 10 U ml⁻¹ IFN- γ , reaching a maximum at 48 h, when 20% of all cells were induced (Fig. 1a). By immunofluorescence microscopy, Ia⁺ cells also became apparent after 18 h of treatment whereas control cultures showed no Ia⁺ cells. Double immunofluorescence microscopy revealed that

Table 1 Induction of Ia antigen expression on glial cell cultures

Treatment of primary glial cell cultures	Ia induction
Control (DMEM with 15% FCS)	—
Control conditioned media (glial or DBT cells)	—
Rat IFN- γ (10^5 U ml $^{-1}$)	+
Rat IFN- γ + anti-rat IFN- γ ($1,000$ NU ml $^{-1}$)	—
Infectious JHM virus (10^3 PFU ml $^{-1}$)	+
Ultraviolet-inactivated JHM virus (10^3 PFU ml $^{-1}$)	+
JHM virus (glial or DBT cell-derived) + anti-rat IFN- γ	+
JHM virus + a non-neutralizing anti-JHM antibody	+
JHM virus + a neutralizing anti-JHM antibody	—

+, Detectable by immunofluorescence microscopy (induction of Ia in at least 2,500–5,000 cells per cm 2); —, undetectable by immunofluorescence microscopy (induction of Ia in 0–10 cells per cm 2). DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum. The stock preparation of recombinant rat IFN- γ contained 1.2×10^5 U ml $^{-1}$ and 3×10^6 U per mg protein. Polyclonal rabbit antiserum to rat IFN- γ , given by Dr van der Meide, contained 1.0×10^5 neutralizing units (NU) ml $^{-1}$. JHM virus was obtained from tissue culture supernatants of cells infected with wild-type JHM murine coronavirus. Virus supernatants were produced from two different sources, primary glial cultures and a cell line permissive for JHM (designated DBT). The amount of virus in the supernatants was determined by titration (as PFU ml $^{-1}$) on DBT cells. Stock virus from DBT cells numbered 2×10^5 PFU ml $^{-1}$ and from primary glial cultures, 2×10^4 PFU ml $^{-1}$, when the cytopathic effect reached 90%. Virus preparations were completely inactivated with ultraviolet light ($2,500 \mu\text{W cm}^{-2}$) for 5 min. Conditioned supernatants from uninfected cultures served as the control for the virus supernatant preparations. Monoclonal antibody directed against the envelope glycoprotein E2 has been described elsewhere⁹. The neutralizing monoclonal antibody was used at a dilution sufficient to neutralize 10^5 PFU ml $^{-1}$. Cultures were treated as indicated for 4 days, stained for Ia using OX6 monoclonal antibody, then examined by fluorescence microscopy as described in Fig. 2 legend. The total number of cells in 10-day cultures was, on average, 10^5 cells cm $^{-2}$.

macrophages as well as a small proportion of the type I astrocyte population were induced to express Ia (Fig. 2d–f).

Treatment of primary glial cell cultures with either infectious or ultraviolet-inactivated JHM virus also induced Ia expression by astrocytes in a dose-dependent manner, peaking at 10^3 plaque-forming units (PFU) ml $^{-1}$ (Table 1); higher concentrations had a toxic effect on the cells. JHM virus at 10^3 PFU ml $^{-1}$ gave the maximum response regardless of the source (Table 1). Immunofluorescence microscopy of cultures treated with ultraviolet-inactivated JHM virus, using a polyclonal rabbit antiserum to JHM virus, confirmed the absence of infected cells throughout the cultures, indicating that there was no JHM virus replication. Fluorescence-activated cell sorting showed that ~10% of all cells in the cultures became Ia-positive (Fig. 1b). Conditioned media from uninfected cultures, diluted correspondingly, had no effect on Ia expression. In contrast to rat IFN- γ , JHM virus induced Ia primarily in the astrocytic cell population (Fig. 2g–i), 90–100% of macrophages remaining negative, as determined by double immunofluorescence microscopy. In addition, the kinetics of induction was distinct from that seen with rat IFN- γ in that noticeable induction required at least 3–4 days of treatment, reaching a peak at 4–7 days. Double immunofluorescence analysis of GFAP and Ia showed that between 90 and 100% of the induced cells were astrocytes (Fig. 2g–i). In cultures treated with infectious JHM virus, the numerous Ia-positive astrocytes induced were apparently uninfected, as revealed by double immunofluorescence of Ia and JHM viral antigen. If indomethacin was added together with JHM virus, almost all the macrophages expressed Ia whereas

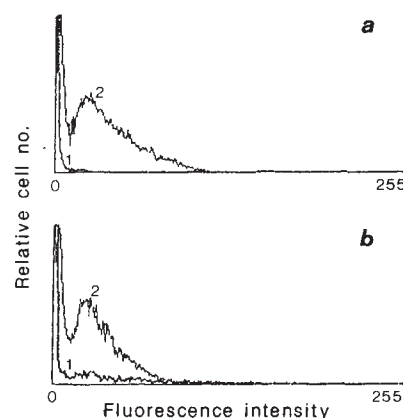


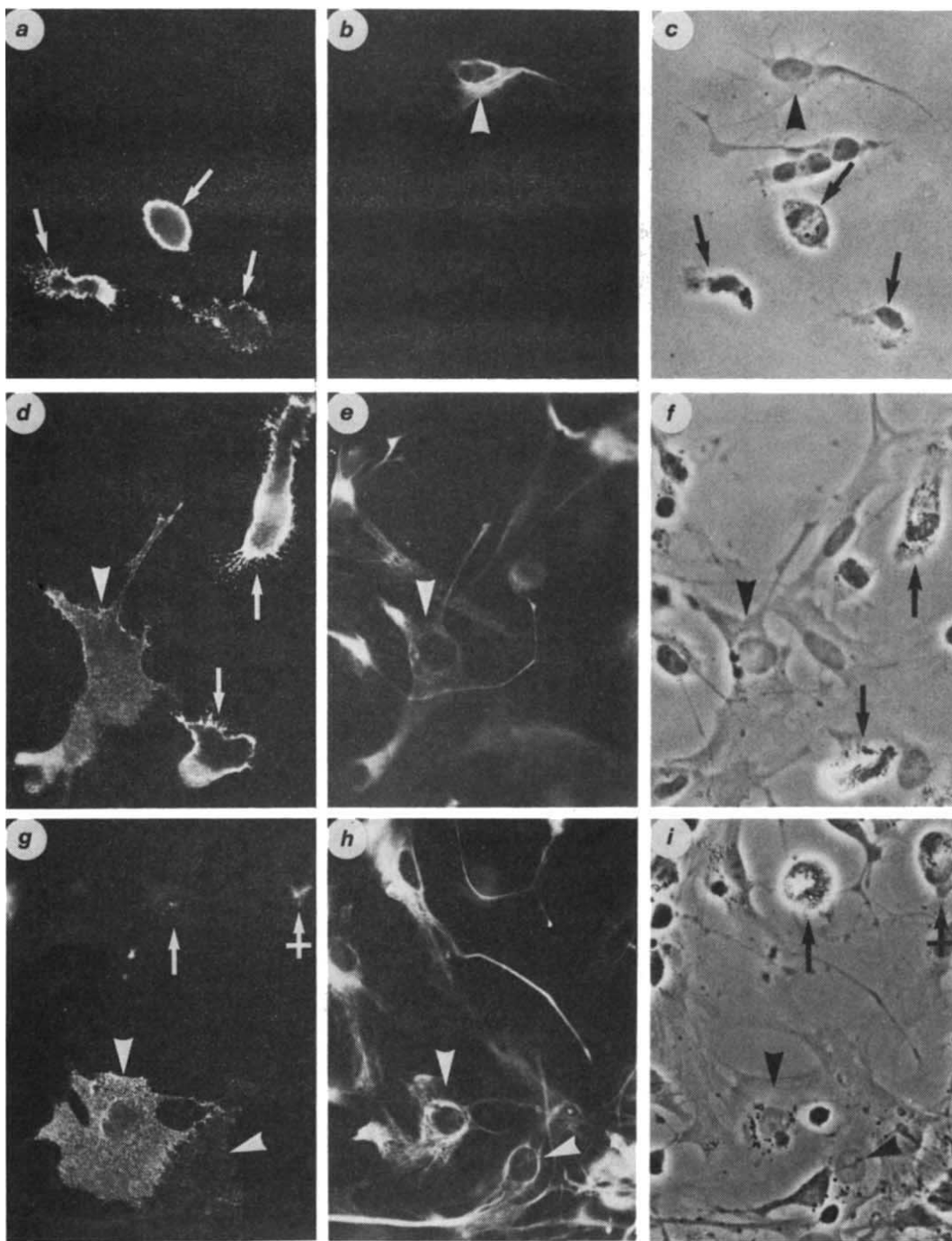
Fig. 1 Analysis of Ia induction using the Epics V fluorescence-activated cell sorter (FACS; Coulter Electronics, Hialeah, Florida). **a**, Cells treated with IFN- γ . Cells were stained by incubation with OX6 primary antibody¹⁸, then with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Curve 1, no pretreatment with recombinant rat IFN- γ ; 2, pretreated for 48 h with 10^5 U ml $^{-1}$ recombinant rat IFN- γ . Approximately 20% of viable cells recovered for FACS analysis were induced to express Ia (between channels 10 and 255). Histogram scale 1:512. **b**, Cells treated with JHM virus. Cells were stained with OX6 primary antibody, then with FITC-conjugated secondary antibody. Curve 1, no pretreatment with JHM; 2, pretreated for 4 days with JHM virus (10^3 PFU ml $^{-1}$). Approximately 10% of all viable cells recovered were induced to express Ia (channels 10–255). Histogram scale 1:256. **Methods.** Viable Lewis primary glial cell cultures were incubated for 1 h with a 1:2 dilution of mouse monoclonal OX6 hybridoma supernatant ($20 \mu\text{g ml}^{-1}$ IgG) in DMEM with 20% normal horse serum (at 4°C). Cultures were rinsed three times, then incubated with a 1:20 dilution of FITC-conjugated rabbit F(ab) $_2$ anti-mouse immunoglobulin (Dakopatts, Denmark), for 30 min. Cultures were rinsed with 0.12 M phosphate buffer pH 7.2, containing 1% bovine serum albumin (BSA), removed from the culture dishes and mechanically dissociated by pipette aspiration. After addition of $0.2 \mu\text{g ml}^{-1}$ propidium iodide, cells were analysed immediately. Gate window of forward angle light scatter (FALS) lay between channels 10 and 255. Gate window for log integral red fluorescence was set for exclusion of non-viable cells stained bright red with propidium iodide. Gate window for log integral green FITC fluorescence (LIGFL) lay between channels 0 and 255 (abscissa). The number of Ia-positive cells was computed by integration from channel 10 to 255 for each sample containing 50,000 cells.

macrophages without virus remained negative; this indicated that the ability of macrophages to express Ia was positively influenced by JHM virus, but that prostaglandins suppressed expression⁷. Astrocytes appeared resistant to such suppression.

To determine whether the JHM virus had a direct effect on astrocytes or whether the effect was due to a secondary signal released by macrophages⁸ or astrocytes themselves, astrocytic cultures depleted of macrophages were tested for their responsiveness to JHM virus. Macrophages were removed by panning of trypsinized primary cultures on hydrophobic plastic; the relatively non-adherent astrocytes remaining in suspension were removed and re-plated. After 4 days of treatment with 10^3 PFU ml $^{-1}$ ultraviolet-inactivated virus, these astrocytes expressed Ia, just as in cultures containing macrophages. Supernatants derived from either pure macrophage cultures or mixed primary cultures, after incubation with inactivated JHM virus, failed to induce Ia on naive astrocytes. This result indicated that secondary soluble factors were not involved in the induction of Ia antigen on astrocytes.

The possibility that Ia induction was the result of virus-induced interferon synthesis in the cultures was also examined. Primary cultures were treated with JHM virus (10^3 PFU ml $^{-1}$, infectious or ultraviolet-inactivated) for 4 days, after which they

Fig. 2 *a-c*, Characterization of major cell types in one microscopic field by double immunofluorescence and phase-contrast microscopy of a 10-day control Lewis primary glial cell culture. *a*, Three Fc receptor⁺ (Fc⁺) macrophages with characteristic microspikes adhering to the substratum (arrows). *b*, One GFAP⁺ astrocyte (arrowhead) with fibrillar staining pattern characteristic of GFAP intermediate filaments. Cells were never double-labelled for Fc receptors and GFAP (see also ref. 15). *c*, Phase-contrast photomicrograph. Note the numerous lysosomal granules within the cytoplasm of Fc⁺ macrophages. Fc⁺ macrophages were found also to ingest large numbers of zymosan particles and to be non-specific esterase-positive, both characteristic features of macrophages. *d-f*, Double immunofluorescence and phase-contrast microscopy of one microscopic field of a 10-day primary glial cell culture treated for 18 h with recombinant rat IFN- γ (10 U ml⁻¹). *d*, Two macrophages (arrows) and one astrocyte (arrowhead) labelled for surface Ia. *e*, GFAP⁺ astrocytes with characteristic GFAP fibrillar staining pattern. Not all GFAP⁺ astrocytes are Ia-positive. The Ia⁺ macrophages in *d* are clearly negative for GFAP staining and can be identified by their characteristic microspikes (*d*) and lysosomal granules (arrows in *f*). *f*, Phase-contrast photomicrograph. The astrocyte indicated by an arrowhead in *d-f* is Ia⁺. *g-i*, Double immunofluorescence and phase-contrast microscopy of one microscopic field of a 10-day primary glial cell culture treated during the previous 4 days with 10³ PFU ml⁻¹ ultraviolet-inactivated JHM virus. *g*, Two astrocytes expressing cell-surface Ia (arrowheads). *h*, GFAP⁺ astrocytes showing a fibrillar staining pattern. The arrowheads in *h* and *i* pinpoint the two Ia⁺ cells that are indicated by arrowheads in *g*, showing strong double immunofluorescence of Ia and GFAP for the same cells. Note that the unlabelled GFAP⁺ astrocytes in *h* are not Ia-positive in *g*. *i*, Phase-contrast photomicrograph. The cell labelled with an arrow contains granules typical of macrophages and is negative for Ia (see *g*) and GFAP (*h*). The other granule-containing macrophage indicated by the crossed arrow shows only weak expression of Ia in *g* at the lower pole of its cell body bearing microspikes. Therefore, GFAP⁺ astrocytes are selectively induced to express Ia while over 90% of macrophages remain Ia⁻. Parallel cultures stained for JHM antigen revealed no infected cells throughout the cultures.



g-i, Double immunofluorescence and phase-contrast microscopy of one microscopic field of a 10-day primary glial cell culture treated during the previous 4 days with 10³ PFU ml⁻¹ ultraviolet-inactivated JHM virus. *g*, Two astrocytes expressing cell-surface Ia (arrowheads). *h*, GFAP⁺ astrocytes showing a fibrillar staining pattern. The arrowheads in *h* and *i* pinpoint the two Ia⁺ cells that are indicated by arrowheads in *g*, showing strong double immunofluorescence of Ia and GFAP for the same cells. Note that the unlabelled GFAP⁺ astrocytes in *h* are not Ia-positive in *g*. *i*, Phase-contrast photomicrograph. The cell labelled with an arrow contains granules typical of macrophages and is negative for Ia (see *g*) and GFAP (*h*). The other granule-containing macrophage indicated by the crossed arrow shows only weak expression of Ia in *g* at the lower pole of its cell body bearing microspikes. Therefore, GFAP⁺ astrocytes are selectively induced to express Ia while over 90% of macrophages remain Ia⁻. Parallel cultures stained for JHM antigen revealed no infected cells throughout the cultures.

Methods. Primary glial cell cultures were established from newborn (1 day postnatal) Lewis rat brain as described previously¹⁶. Six days after plating, at which stage the cultures were treated with IFN- γ or JHM virus, three distinct cell populations were present: type I astrocytes, macrophages and A2B5⁺ precursors to both type II astrocytes and galactocerebroside-positive oligodendrocytes¹⁷. Staining of Fc receptors was achieved by incubating live cultures with a 1:100 dilution of normal mouse serum, followed by goat anti-mouse IgG conjugated to TRITC (Zymed, California), at 4 °C (ref. 15). After fixation with 2% formaldehyde and permeabilization with 0.25% Triton X-100, GFAP filaments were stained using a polyclonal rabbit IgG directed against GFAP (Dakopatts, Denmark) diluted 1:250, followed by goat anti-rabbit IgG conjugated to FITC (Zymed). Staining of rat Ia and GFAP was as for Fc receptors and GFAP. A mouse monoclonal antibody directed against rat Ia (designated OX6; given by Dr D. W. Mason) was diluted to 20 μ g ml⁻¹ IgG from hybridoma supernatant. As a control for the OX6 monoclonal antibody which is of the IgG1 subclass, two different mouse monoclonal IgG1 antibodies against unrelated antigens were tested and found to be negative. Staining of JHM virus antigen was performed as for GFAP, using a rabbit IgG fraction directed against JHM, diluted to 20 μ g ml⁻¹ IgG.

were challenged with vesicular stomatitis virus (VSV; 100 PFU ml^{-1}). One day after infection, titrations of VSV released showed no reduction of PFU ml^{-1} compared with the control. In addition, both untreated and JHM virus-treated cultures were totally destroyed by VSV, indicating an absence (or insufficient levels) of interferon(s). In addition, the application of a polyclonal rabbit antiserum to rat IFN- γ in conjunction with JHM virus obtained from either infected DBT cells or primary glial cell cultures did not block or reduce Ia induction. The concentration of rabbit anti-rat IFN- γ used effectively eliminated the Ia-inducing capacity of 10 U ml^{-1} recombinant rat IFN- γ at 48 h post-treatment (Table 1).

The Ia antigen-inducing capacity of JHM appears to depend on direct interaction of JHM virus with the astrocytes. The envelope glycoprotein E2 would be the most appropriate candidate for such virus-cell interactions as it is responsible for binding of the virus to susceptible cells and for virus-induced cell fusion⁹. To test this notion, cultures were treated with JHM virus in conjunction with a neutralizing monoclonal antibody directed against the E2 glycoprotein. A non-neutralizing antibody to JHM virus was used as a control. After 4 days of treatment, cultures exposed to non-neutralizing antibody showed a typical pattern of Ia induction, whereas the neutralizing antibody to E2 totally blocked JHM virus induction of Ia (Table 1); this indicates that virus-cell binding mediated via the E2 glycoprotein of JHM has an important role in the induction of Ia on astrocytes.

Here, we have described the ability of a neurotropic virus to induce Ia molecules on astrocytes, and we have presented evidence that JHM virus exerts its effects on astrocytes through direct interaction. This phenomenon is independent of viral replication in astrocytes as ultraviolet-inactivated virus is effective in inducing Ia antigen. Moreover, a monoclonal antibody to the E2 glycoprotein which blocks virus binding to susceptible cells⁹, also blocks the induction of Ia antigen. These observations suggest that either binding of the virus to the surface of astrocytes through specific cell-surface receptors or phagocytosis of the virus particles initiates a series of events in astrocytes leading to Ia expression. The mechanism by which JHM virus induces Ia expression is unknown; it is conceivable that viral glycoproteins may act on particular target cells in the induction of Ia in a similar way to that described for bacterial endotoxin¹⁰.

The present results are particularly interesting as IFN- γ released by T lymphocytes is thought to be indispensable in the induction of Ia antigen on certain antigen-presenting cells^{11,12}, including astrocytes¹⁻³. Astrocytes could be especially effective antigen-presenting cells in the brain owing to their ubiquity and their ability to phagocytose, process and present antigen³. In the case of virus invasion of the CNS, this cell population may play an important part in mounting an immune response to effectively control the viral infection. On the other hand, high constitutive levels of Ia expression might carry the risk of inappropriate presentation of self antigens, as is thought to occur in autoimmune processes directed against the thyroid gland¹³. This phenomenon may have special relevance to brain antigens and Ia-expressing astrocytes as the development of immune tolerance to self brain antigens, including myelin, may be hampered by the blood-brain barrier. The induction of Ia on astrocytes by JHM virus probably has a role in the JHM virus-induced chronic demyelinating disease of Lewis rats, which involves induction of myelin basic protein-specific T lymphocytes⁶. The phenomenon reported here may represent a general feature of virus-astrocyte interactions and may have wider implications for human neurotropic viruses and the induction of immunologically mediated chronic demyelinating diseases¹⁴.

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1. Wong, G. H. W., Clark-Lewis, I., Harris, A. W. & Schrader, J. W. *Eur. J. Immun.* **14**, 52-56 (1984).
2. Hirsch, M. R., Wietzerbin, J., Pierres, M. & Goridis, C. *Neurosci. Lett.* **41**, 199-204 (1983).
3. Fontana, A., Fierz, W. & Wekerle, H. *Nature* **307**, 273-276 (1984).
4. Wege, H., Siddell, S. & ter Meulen, V. *Curr. Topics Microbiol. Immun.* **99**, 165-189 (1982).
5. Wege, H., Watanabe, R. & ter Meulen, V. in *Progress in Clinical & Biological Research. Experimental Allergic Encephalomyelitis: A Useful Model for Multiple Sclerosis* (eds Alvord, E. C., Kies, M. W. & Suckling, A. J.) (Liss, New York, 1984).
6. Watanabe, R., Wege, H. & ter Meulen, V. *Nature* **305**, 150-153 (1983).
7. Snyder, D. S., Beller, D. I. & Unanue, E. R. *Nature* **299**, 163-165 (1982).
8. Walker, W. B., Maino, V., Sanchez-Lanier, M., Warner, N. & Stewart, C. J. *exp. Med.* **159**, 1532-1547 (1984).
9. Wege, H., Dörries, R. & Wege, H. *J. gen. Virol.* **65**, 1931-1942 (1984).
10. Monroe, J. G. & Cambier, J. C. *J. exp. Med.* **158**, 1589-1599 (1983).
11. Steeg, P. S., Moore, R. N., Johnson, H. M. & Oppenheim, J. J. *J. exp. Med.* **156**, 1780-1793 (1982).
12. Steeg, P. S., Johnson, M. & Oppenheim, J. J. *J. Immun.* **129**, 2402-2406 (1982).
13. Londei, M., Lamb, J. R., Bottazzo, G. F. & Feldmann, M. *Nature* **312**, 639-641 (1984).
14. Traugott, U., Scheinberg, L. C. & Raine, C. S. *J. Neuroimmun.* **8**, 1-14 (1985).
15. Raff, M. C., Fields, K. L., Hakomori, S. I. & Mirsky, R. *Brain Res.* **174**, 283-308 (1979).
16. Massa, P. T. & Mugnaini, E. *Neuroscience* **14**, 695-709 (1985).
17. Raff, M. C., Miller, R. H. & Noble, M. *Nature* **303**, 390-396 (1983).
18. McMaster, W. R. & Williams, A. F. *Eur. J. Immunol.* **9**, 426-433 (1979).

Major reorganization of immunoglobulin V_H segmental elements during vertebrate evolution

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In mammals, the immunoglobulin heavy-chain variable region (V_H) locus is organized in a linear fashion; individual V_H , diversity (D_H), joining (J_H) and constant (C_H) region segments are linked in separate regions¹. During somatic development, coding segments flanked by characteristic short recombination signal sequences, separated by intervening sequence regions that may exceed 2,000 kilobases (kb), are recombined. Combinatorial joining of different segments as well as imprecision in this process contribute to the diversity of the primary antibody response; subsequent mutation further alters functionally rearranged genes. This basic somatic reorganization mechanism is shared by six major families of genes encoding antigen receptors². Previously, we have shown that multiple germline genes and mammalian-like recombination signal sequences are associated with the V_H gene family of *Heterodontus francisci* (horned shark), a primitive elasmobranch³. Studies presented here demonstrate that segmental reorganization involving mammalian-like D_H and J_H segments occurs in the lymphoid tissues of this species. In marked contrast to the mammalian system, we find multiple instances of close linkage ($\sim 10 \text{ kb}$) between individual V_H , D_H , J_H and C_H segments. This unique organization may limit combinatorial joining and be a factor in the restricted antibody response of this lower vertebrate^{4,5}.

A *Heterodontus* spleen poly(A)⁺ messenger RNA-complementary DNA library was constructed in the vector λ gt11 (ref. 6) and screened with a nick-translated probe that complements the entire coding region of *HXA*, a *Heterodontus* germline V_H gene³. Multiple positive hybridizing plaques were detected and the structure of one of these, *HC-3*, is illustrated in Fig. 1. Regions exhibiting high degrees of nucleotide identity

Fig. 1 Comparison of the nucleotide sequences of *HC-3* with the framework (FR)1, complementarity determining (CDR)1, FR2, CDR2 and FR3 segments of *HXIA*, a germline *Heterodontus* V_H gene, and with the human *J3* (J) segment and human C_{μ} 1 (C) constant region. Nucleotide sequence identities are indicated by upper-case letters, non-identities by lower-case letters. Predicted identity of amino acids is indicated by larger type and non-identities are indicated by smaller type. An insertion (-) has been introduced in FR2 to maximize homology with the *HXIA* prototype 5' and 3' to this position; 'X' has been introduced in the amino-acid line corresponding to this ambiguity. The sequence through the region has been confirmed on both strands by dideoxynucleotide chain termination as well as by chemical sequencing of shorter restriction fragments bounding this region. If the deletion in *HC-3* (by comparison with *HXIA* and mammalian genes) is not a cloning artefact, a number of highly conserved N-terminal positions would be affected and presumably would not yield a functional peptide. Cleavage of the 319-bp *Eco*RI fragment of *HC-3* at the *Hae*III site ($Gg^{\nabla}cC$) within CDR2 defines a V_H -specific probe ($\square$); a *Hae*III cleavage at the end of FR3 ($gG^{\nabla}cC$) and *Pst*I cleavage ($CTgCA^{\nabla}G$) between J and C permitted the cloning of *D/J_H* ($\boxtimes$) and C_H ($\blacksquare$) segments in M13 replicative forms; the sequences of the single-strand preparations were confirmed.

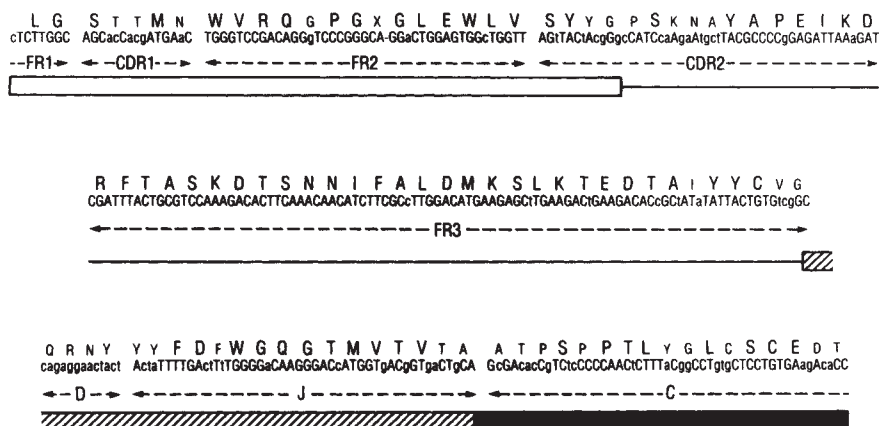
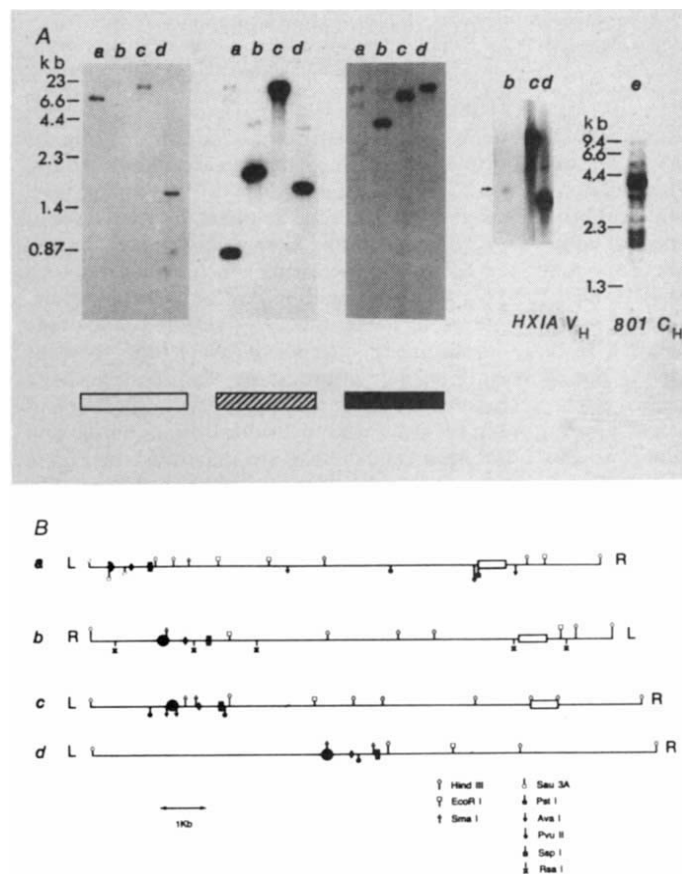


Fig. 2 **A**, Filter hybridization of *Heterodontus* DNA with single-strand probes complementing different immunoglobulin V_H segmental elements. DNA (250 ng) from each *Heterodontus* phage clone was digested to completion with *Pst*I, fractionated on a 1% agarose gel and transferred to nitrocellulose. Single-strand probes were prepared from subclones of the portions of *HC-3* indicated in Fig. 1, using an M13 probe-primer method¹⁰. Hybridization was at 65 °C for 16 h as described previously³, using $\sim 1 \times 10^6$ c.p.m. per 10 ml of each probe, indicated by shading, which corresponds to the specific genetic regions indicated in Fig. 1. The blots were washed three times at room temperature and three times at 52 °C in 150 mM NaCl, 15 mM sodium citrate, 0.1% SDS and 0.05% sodium pyrophosphate. A 16-h autoradiographic exposure is shown. Lanes a, λ HXIA; lanes b, λ 801; lanes c, λ 821; lanes d, λ 923. The inset is included to show the weak hybridization observed when λ 801 (lane b) (compared with λ 821 (lane c) and λ 923 (lane d)) was hybridized with the complete V_H region of λ HXIA ($HXIA V_H$) labelled by nick translation. The varying levels of hybridization intensity are consistent with the use of single-strand probes and with J_H possessing the highest degree of uninterrupted nucleotide sequence identity between different isolates. The additional inset represents a Southern blot of 10 μ g of *Pst*I-digested *Heterodontus* genomic DNA (lane e) hybridized with a C_H -specific probe derived from λ 801 ($801 C_H$). The ~ 400 -bp probe (nick-translated to $\sim 1.5 \times 10^8$ c.p.m. μ g⁻¹) was hybridized in conditions described previously³. Multiple hybridizing components are evident and the size of the principal hybridizing component(s) was identical in parallel genomic and λ 801 digests; the specificity of C_H hybridization has been confirmed by nucleotide sequencing and comparison with the prototypic *Heterodontus* C_H sequence (Fig. 1). **B**, Physical maps establishing the linkage relationships between segments hybridizing with V_H , J_H (the *D* component of *HC-3* (Fig. 1) did not hybridize and its location was determined by sequencing) and C_H probes relative to the left (L) and right (R) arms of the bacteriophage λ vector— λ 801 (a), λ 821 (b) and λ 923 (c)—which were isolated from a germline library by hybridization with a *D/J_H* probe. λ HXIA (d) was isolated by hybridization with a V_H probe³. The restriction endonucleases used to establish the primary map relationships are indicated above the line; those used in fine mapping (usually in conjunction with nucleotide sequencing) are indicated below the line and are indicated only at those positions which facilitated localization of a specific genetic region. The positions of V_H ($\bullet$), D_H ($\blacklozenge$), J_H ($\blacksquare$) and C_H ($\square$) are indicated. $\blacktriangleright$ Indicates the presence of a weakly hybridizing restriction fragment, displaying sequence homology to V_H . Using a specific probe for the left arm of λ , it was possible to show that a *Sau*3A site within the 3' portion of a V_H coding region is at the (L) end of the genomic insert. The 3' recombination signal sequence of this gene is CACTGTG-23-bp spacer-ATGAATAAA. Note that the specific C_H probe used complements only the first ~ 50 bp of the constant region (Fig. 1) and the overall dimensions of the symbol used are not intended to represent the actual genetic boundaries of these segments.



with the homologous V_H probe as well as with human J_H3 (ref. 7) and $C_{\mu}1$ (ref. 8) sequences are evident. From its placement, a nucleotide segment between the V_H and J_H appears to correspond to a *D*- or *N* (ref. 9)-like sequence. The locations of *Hae*III and *Pst*I restriction sites permitted the convenient derivation of fragments corresponding to V_H , *D/J_H* and C_H

regions which were cloned in M13mp10/11 replicative forms. An oligonucleotide probe primer¹⁰ was used to generate high-specificity probes complementing the three regions indicated in Fig. 1.

As a first step in characterizing the germline structures of the segmental components, a *Heterodontus* genomic DNA library

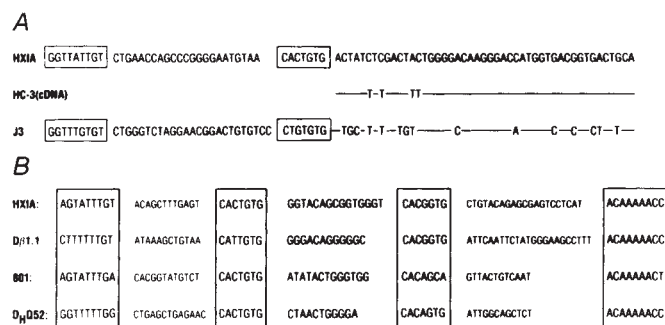


Fig. 3 **A**, Nucleotide sequence of the J_H region of *HXIA* including the putative 5' recombination signal sequence (boxed) compared with the corresponding sequence (putative coding region only) of *HC-3* (see Fig. 1) and *J3*, a human germline J_H gene, including its recombination signal sequence (boxed)⁷. A dash indicates sequence identity with the *HXIA* J_H . **B**, Nucleotide sequence comparisons of: the putative D_H region of *HXIA* (Fig. 2B, d); $D_{\beta}1.1$, a D region associated with the murine β T-cell receptor complex¹²; *801*, the putative D_H region of λ 801 (Fig. 2B, a); and *DQ52*, a germline human D_H segment^{7,17}. Recombination signal elements are boxed; spacer segments are indicated by smaller type; other positions (large font) represent presumed coding segments. A sequence homology between six consecutive nucleotides in D_H Q52 and *801* is apparent.

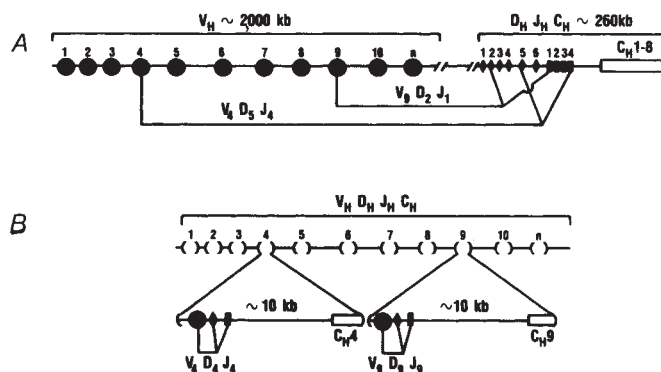


Fig. 4 **A**, A schematic depiction (number and position of segments not based on absolute physical map data) of the mammalian (mouse) V_H locus illustrating the linear relationships of V_H , D_H , J_H and C_H components. In the mouse and presumably other mammals, 100 V_H segments may occupy ~2,000 kb^{19,20}; the D_H , J_H and C_H segments occupy ~260 kb²¹ and the physical distance between the two regions may be ~2,000 kb²² (in human). In both the mouse and human, *DQ52* is located within the D_H cluster (not illustrated). Two combinatorial joinings are illustrated, each involving >2,000 kb of DNA. **B**, In the elasmobranch (shark), V_H , D_H , J_H segments occur within ~1.3 kb and with the C_H segment occupy ~10 kb. While the presence of multiple individual clusters has been established, the ~12-kb length of the λ genomic inserts precludes establishing linkage relationships between clusters, which may be neither contiguous nor necessarily on the same chromosome. The two joinings are shown as being non-combinatorial, which we suggest may be a consequence of this specific type of gene arrangement.

was screened, under moderately stringent conditions³, using the D/J_H probe. Multiple hybridizing clones were identified and five isolates, shown to be unique by restriction mapping, were subjected to further characterization. Southern blot analyses of these genomic D/J_H^+ phage (~12 kb average insert size) showed that four contained restriction fragments which hybridized with the V_H , D/J_H and C_H specific probes. Figure 2A shows hybridization patterns of three of these and a V_H -selected clone (see below). Initially, these unexpected results were thought to be due to the abundant presence of processed V_H pseudogenes in this species. Restriction maps for the insert regions of three of these clones, inferred from the hybridization patterns and confirmed by additional sequencing, are illustrated in Figure 2B, a-c and a unit $V_H \leftrightarrow C_H$ distance of ~10 kb is evident. The single isolate (clone 802) that hybridized only with the D/J_H probe was found, by sequence analyses, to represent an unusual J_H pseudogene located ~500 base pairs (bp) 3' to a typical (see below) D segment. In a similar manner, when 14 unique *Heterodontus* genomic clones that had been selected with a heterologous (murine) V_H probe³ were blotted and screened with the D/J_H and C_H probes, 11 also hybridized with the D/J_H probe and, of these, 2 were found also to hybridize with the C_H probe. These distributions of hybridizing segments are consistent with the use of a V_H probe in the initial selection, an ~10-kb linkage distance and the (~12-kb) λ insert sizes. Restriction mapping has shown that V_H , D/J_H and C_H hybridizing segments are not joined in the genomic configuration. Furthermore, a 350-bp probe complementing the region 3' to the recombination signal sequence of *HXIA*³ was found to hybridize with 9 out of 10 of the λ isolates; if V_H had recombined, this region would have been eliminated. The single isolate, λ 821, that did not hybridize with this probe was found to have typical recombination signal sequences (see below) and may represent a member of a V_H subset lacking sufficient 3' homology with *HXIA*. The unexpected complexity of the C_H gene family, suggested by the phage mapping experiments, was indicated further by Southern blotting of *Heterodontus* genomic DNA using a nick-translated C_H -specific probe derived from λ 801 (Fig. 2A).

HXIA, the prototypic elasmobranch V_H gene that appears to be transcribed in the *Heterodontus* spleen³, was among the group of V_H -selected phage that hybridized with the D/J_H probe, and its restriction map, established by both hybridization and DNA sequencing, is shown in Fig. 2B, d. The predicted coding

sequence of the *HXIA* J_H segment (Fig. 3A) is 91% identical to that of *HC-3*, the rearranged gene (Fig. 1). If recombined in a mammalian-like manner, this sequence would encode a peptide segment differing from *HC-3* by only two amino acids. The 3' sequences of both of these genes are more closely related to mammalian J_H segments^{7,11} than are their respective 5' sequences. The J_H recombination signal 7-mer differs from that of human *J3* but is identical to the mammalian J_H consensus 7-mer. The 9-mer is T>G-rich and the spacer is of characteristic (~23 bp) length^{2,3,11}. The nucleotide sequence between the recombination signal sequences found 3' of the *HXIA* V_H (ref. 3) and 5' of the J_H segment was determined and a segment located ~700 bp 5' of J_H possesses several characteristics of a germline D segment (Fig. 3B): (1) 3' Recombination signal sequence 7- and 9-mers are identical to those of $D_{\beta}1.1$, a T-cell receptor D gene¹², although the V_H segment clearly is of the higher vertebrate type. (2) The 5' recombination signal sequence 7-mer, CACTGTG, is identical to the D_H consensus, and is located 12 bp downstream from a 5' T>G-rich 9-mer¹. The length of the 3' spacer region (~21 bp) is less than that of $D_{\beta}1.1$ (~23 bp), and is not typical of mammalian immunoglobulin D_H regions (~12 bp) or even of other elasmobranch D_H regions (~12 bp) such as that identified 3' of the *801* J_H segment (Fig. 3B), or the *801*-like D segments of λ 821, λ 802 (see above) and λ 923 (not illustrated). Although the homology of the *HXIA* D to mammalian and other elasmobranch D_H segments is apparent, the manner in which it would functionally recombine, while adhering to the 23/12 spacing rule², is unclear. It remains possible that the *HXIA* isolate is a nonfunctional member of a closely related V_H gene subset suggested in earlier work³.

The possible genetic consequences of the novel linkage pattern are not understood; however, it is predicted to be a ubiquitous feature of the elasmobranch V_H system. The organization may favour joining of adjacent V_H , D_H , J_H and constant-region partners. Junctional (joining) diversity presumably would introduce some degree of somatic difference; however, combinatorial diversity may be a less important aspect of gene rearrangements in this species than in mouse and human. The large number of

germline V_H and different D_H and J_H segments presumably would contribute to the significant antibody repertoire of *Heterodontus*. An absence or reduced degree of combinatorial diversity may account for the remarkable lack of inter-individual variation associated with the short- and long-term-specific immune response of this species^{4,5}. Recently, it has been shown that combinatorial diversity dominates the secondary immune response to 2-phenyl-5-oxazolone, which is accompanied by increases in antigen-binding affinity and differences in fine specificity¹³. In terms of both of these functional parameters, *Heterodontus* lacks a secondary immune response even after long periods of hyperimmunization. In a poikilothermic species whose cell proliferation kinetics may be unsuitable for generating and selecting an extensive repertoire of closely related antigen-binding specificities¹⁴, close linkage and presumably reduced recombination between different clusters of V_H , D_H , J_H and C_H segments may ensure efficient formation of certain antibodies; natural selection could act more efficiently on the different segmental elements contained in a closely linked cluster (without combinatorial joining) than on segments distributed over large chromosomal distances (with combinatorial joining). If combinatorial diversity is not necessarily advantageous, why is the gene segmented and recombined in this manner? Perhaps the rearrangement process has more to do with eventual regulation of the response through idiotope recognition than with the creation of unique combining specificities. The biological consequences of junctional joining on regulatory idiotope expression are known¹⁵ and include examples of *D*-region changes that alter idiotypic but not antigen-binding specificities¹⁶.

The difference in the organization of the elasmobranch V_H and mammalian V_H (and other *V*) receptor systems is extreme and a hypothetical schematic comparison is given in Fig. 4. The elasmobranch linkage pattern may be representative of a common, ancestral gene family. Although the size of the segmented gene family is unknown, the presence of multiple constant-region segments suggested in these studies raises interesting questions as to their interrelationship and possible functions. A catastrophic loss or successive inactivation of members of this multigene family may have occurred during the vertebrate radiations, as selection favoured a system in which greater somatic variation was introduced. *DQ52*, located ~700 bp 5' to J_H coding regions in human and mouse^{7,17}, may be a modern, partial vestige of the elasmobranch-like organization. Alternatively, the gene arrangement may be a unique adaptation of this lower vertebrate. Although the close linkage of the shark V_H , D_H , J_H and C_H resembles the arrangement of the segmental components of the single, functional avian (chicken) λ light-chain gene¹⁸, it remains to be seen whether the arrangement is characteristic of other lower vertebrate *V* genes. Based on this elasmobranch-mammal comparison, the evolution of the V_H gene family appears to have involved dramatic changes in the chromosomal organization of the individual genetic segments rather than in their numbers or basic mechanisms of recombination.

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1. Tonegawa, S. *Nature* **302**, 575-581 (1983).
2. Hood, L., Kronenberg, M. & Hunkapiller, T. *Cell* **40**, 225-229 (1985).
3. Litman, G. W. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 2082-2086 (1985).
4. Makela, O. & Litman, G. W. *Nature* **287**, 639-640 (1980).
5. Litman, G. W., Stolen, J. S., Sarvas, H. O. & Makela, O. *J. Immunogenet.* **9**, 465-474 (1982).
6. Young, R. A. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1194-1198 (1983).
7. Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. *Cell* **27**, 583-591 (1981).
8. Rabbitts, T. H., Forster, A. & Milstein, C. P. *Nucleic Acids Res.* **9**, 4509-4524 (1981).
9. Alt, F. W. & Baltimore, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4118-4122 (1982).
10. Hu, N.-I. & Messing, J. *Gene* **17**, 271-277 (1982).
11. Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. *Cell* **19**, 981-992 (1980).
12. Siu, G. *et al. Nature* **311**, 344-350 (1984).
13. Berek, C., Griffiths, G. M. & Milstein, C. *Nature* **316**, 412-418 (1985).
14. Du Pasquier, L. *Nature* **296**, 311-313 (1982).

15. Pollok, B. A., Kearney, J. F., Vakil, M. & Perry, R. P. *Nature* **311**, 376-379 (1984).
16. Radbruch, A. *et al. Nature* **315**, 506-508 (1985).
17. Sakano, H., Kurosawa, Y., Weigert, M. & Tonegawa, S. *Nature* **290**, 562-565 (1981).
18. Reynaud, C.-A., Anquez, V., Dahan, A. & Weill, J.-C. *Cell* **40**, 283-291 (1985).
19. Brodeur, P. H. & Riblet, R. *Eur. J. Immun.* **14**, 922-930 (1984).
20. Honjo, T. & Habu, S. A. *Rev. Biochem.* **54**, 803-830 (1985).
21. Wood, C. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3030-3034 (1983).
22. Johnson, M. J., Natali, A. M., Cann, H. M., Honjo, T. & Cavalli-Sforza, L. L. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7840-7844 (1984).

A chromosome 14 inversion in a T-cell lymphoma is caused by site-specific recombination between immunoglobulin and T-cell receptor loci

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Specific chromosomal aberrations are associated with specific types of cancer (for review see ref. 1). The distinctiveness of each association has led to the belief that these chromosomal aberrations are clues to oncogenic events or to the state of differentiation in the malignant cell type²⁻⁶. Malignancies of T lymphocytes demonstrate such an association characterized most frequently by structural translocations or inversions of chromosomes 7 and 14 (refs 7-9). Analyses of these chromosomally marked tumours at the molecular level may therefore provide insight into the aetiology of the cancers as well as the mechanisms by which chromosomes break and rejoin. Here we report such an analysis of the tumour cell line SUP-T1 derived from a patient with childhood T-cell lymphoma carrying an inversion of one chromosome 14 between bands q11.2 and q32.3, that is¹⁰, inv(14)(q11.2; q32.2). These are the same chromosomal bands to which the T-cell receptor α -chain^{11,12} (14q11.2) and the immunoglobulin heavy-chain locus¹³ (14q32.3) have been assigned. Our analysis reveals that this morphological inversion of chromosome 14 was mediated by a site-specific recombination event between an immunoglobulin heavy-chain variable region (Ig V_H) and a T-cell receptor (TCR) α -chain joining segment (TCR J_α). *S*₁ nuclease analysis shows that this hybrid gene is transcribed into poly(A)⁺ RNA.

We began our analysis by studying the organization and structure of the TCR α -chain locus in the SUP-T1 cell line. The germline genomic structure of the TCR α -chain locus comprises a single constant region (C_α) preceded 5' by several joining segments (J_α) dispersed over at least 30 kilobases (kb)¹⁴. The variable regions (V_α) reside at an unknown distance more centromeric to the TCR C_α locus within the same chromosomal band^{15,16}. Using DNA probes containing germline TCR J_α regions spanning 15 kb 5' of TCR C_α , two distinct TCR C_α -linked rearrangements were detected in this cell line. Probe A, from the region 5 kb 5' of TCR C_α , demonstrated a rearrangement seen as a novel 8-kb band on *Hind*III digestion of SUP-T1 genomic DNA (Fig. 1a). Germline 4.3- and 3.6-kb bands were identified with this probe. A second TCR J_α -containing probe, probe B, derived from a region 15 kb farther upstream of TCR C_α , identified a second rearrangement, a novel 2.5-kb band on *Eco*RI-digested genomic DNA (Fig. 1b). With probe B, no germline band was seen in SUP-T1, suggesting that this sequence had been deleted from the allele which contained the more 3' rearrangement. A germline probe taken from a region 2.5 kb farther 5' did not detect any homologous sequences in SUP-T1

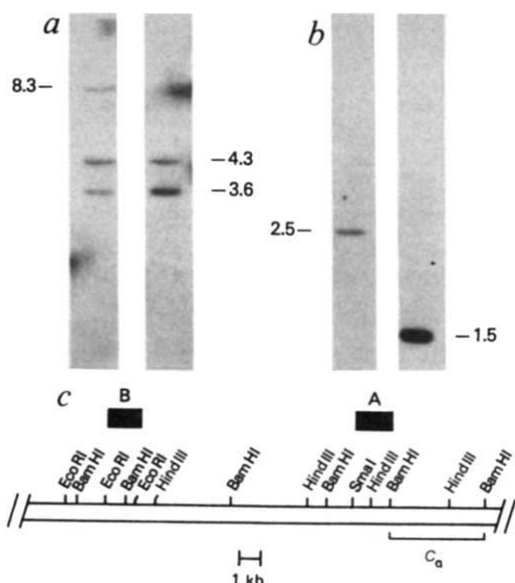


Fig. 1 Rearrangement of two TCR C_α -linked J_α fragments in the T-cell lymphoma cell line SUP-T1. *a*, Southern transfers³⁸ of *Hind*III-digested genomic DNAs (10 μ g per lane) from SUP-T1 (left) or peripheral blood leukocytes (right) were hybridized to a ³²P-labelled DNA probe derived from a 2-kb *Bam*HI-*Sma*I TCR J_α region containing fragment 5 kb 5' of the TCR C_α region (probe A in *c*). The transfers were washed in 0.1 $\times$ SSC, 0.1% SDS at 52 °C and visualized by autoradiography. Germline 4.3- and 3.6-kb bands are seen in both lanes. In addition, a rearranged 8.3-kb band is seen in the SUP-T1 digest. *b*, Southern transfers of *Eco*RI-digested genomic DNAs (10 μ g per lane) from SUP-T1 (left) or peripheral blood leukocytes (right) were hybridized to a ³²P-labelled DNA probe derived from a 1.5-kb *Eco*RI TCR J_α -containing fragment 15 kb 5' of the TCR C_α region (probe B in *c*). The transfers were washed and autoradiographed as described above. A single 2.5-kb rearranged band is seen in the SUP-T1 digest in contrast to the single 1.5-kb germline band in the control lane. *c*, Diagram of the TCR α -chain germline locus.

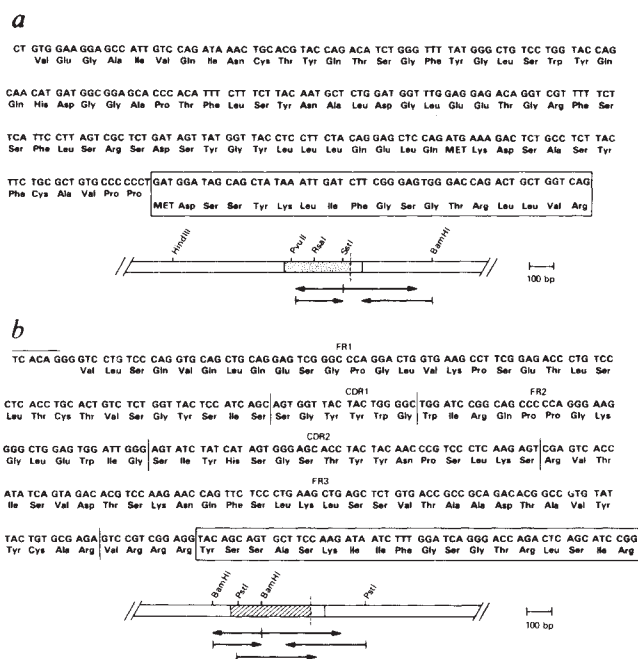


Fig. 2 *a*, Nucleotide sequence and amino-acid translation of the coding sequence of a TCR V_α - J_α rearranged gene from SUP-T1. The TCR J_α sequence is boxed. A possible frameshift occurs at the TCR V_α - J_α junction. The sequencing strategy is shown underneath. Stippled bar, the TCR V_α coding region; open bar represents TCR J_α . A vertical dotted line indicates the position of the V-J junction. *b*, Nucleotide sequence and amino-acid translation of the coding sequence of an Ig V_H -TCR J_α rearranged gene from SUP-T1. The TCR J_α sequence is boxed. Framework (FR) and complementarity-determining (CDR) regions are indicated. The sequencing strategy is shown underneath. $\blacksquare$, Ig V_H coding region; $\square$, TCR J_α . A vertical dotted line indicates the V-J junction. Sequencing was performed by the M13 dideoxy method of Sanger³⁹.

DNA, indicating that this region was deleted in both alleles (data not shown).

The 5'-most C_α -linked J_α rearrangement was cloned by screening a partial *Mbo*I SUP-T1 genomic DNA library with probe B. A comparison with germline DNA allowed the site of recombination to be localized. Sequence analysis (Fig. 2*a*) demonstrated that this rearrangement represents a site-specific recombination between a TCR α -chain variable region and a TCR J_α segment. Amino-acid translation of this sequence indicates a possible frameshift at the point of V-J joining, as is commonly seen in immunoglobulin gene rearrangements (for review see ref. 17).

The other TCR J_α rearrangement was cloned from size-fractionated *Hind*III-digested SUP-T1 genomic DNA; comparison with the germline TCR J_α map allowed precise localization of the region in which recombination had occurred. In this case, however, sequence analysis (Fig. 2*b*) did not reveal the conventional V_α - J_α joining, but instead a site-specific recombination event that joined a completely intact immunoglobulin heavy-chain variable region (Ig V_H) to the TCR J_α segment. Translation of this sequence shows that the Ig V_H -TCR J_α join has an open reading frame for the complete coding region. There is complete amino-acid sequence homology between the framework regions 1, 2 and 3 of this variable-region gene and sequenced proteins of Ig V_H subgroup II (ref. 18). In contrast, there is no extensive homology between this gene and TCR V_α genes sequenced by ourselves or others¹⁹.

Numerous groups have definitively localized the TCR α -chain gene to 14q11.2 (refs 11, 12) and the immunoglobulin heavy-chain variable and constant regions to 14q32.3 (refs 13, 20 and

I.R.K., C. C. Morton and J. V. Ravetch, unpublished data). The orientation of these loci is also known: variable regions are telomeric of constant regions in the immunoglobulin genes²¹ and variable regions centromeric of constant regions in the T-cell receptor α -chain locus^{15,16}. Thus, Ig V_H -TCR J_α recombination would result in chromosomal inversion (Fig. 3), precisely the morphological chromosomal aberration seen in this cell line. Chromosomal mapping studies in analogous systems support this model^{15,16}.

We examined possible transcriptional activity of both TCR V_α - J_α and Ig V_H -TCR J_α rearrangements by *S*₁ nuclease analyses. Single-stranded ³²P-labelled M13 probes complementary to the TCR V_α and Ig V_H genes were prepared and hybridized to total and poly(A)⁺-enriched RNA from SUP-T1 and control cells and subsequently digested with *S*₁ nuclease²². Because the TCR V_α probe contains only TCR V_α coding sequence (Fig. 4*a*), active transcription of this gene should afford full-length protection of this probe. *S*₁ analysis of SUP-T1 RNA revealed a 200-bp protected fragment consistent with transcription of this TCR V_α .

The 270-bp Ig V_H probe contained 120 bp of Ig V_H coding region. RNA from SUP-T1 protected a 120-bp fragment, consistent with specific transcription of this Ig V_H (Fig. 4*b*). The Ig V_H transcript was enriched in the poly(A)⁺ fraction. Additional *S*₁ nuclease analysis using a probe including the 3' part of Ig V_H , the TCR J_α and intervening sequence, has demonstrated that Ig V_H and TCR J_α are transcribed on the same message. The Ig V_H -TCR J_α junction remains in-frame and, at least theoretically, subsequent translation of this hybrid message into protein would be possible.

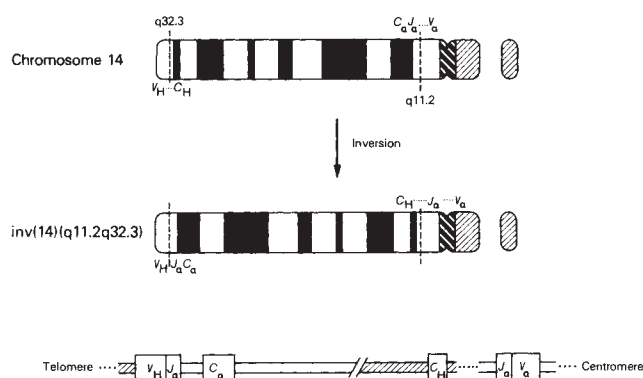


Fig. 3 A model for inversion of chromosome 14 via Ig V_H -TCR J_α site-specific recombination. The chromosomal band localization of the immunoglobulin and T-cell receptor loci and their orientations are shown in the upper idiogram of a normal chromosome 14. Site-specific recombination between an immunoglobulin heavy-chain variable region (V_H) and a T-cell receptor α -chain joining segment (J_α) results in an inversion of chromosome 14 at bands q11.2 and q32.3 (vertical dotted lines), as shown in the lower idiogram. The additional TCR V_α - J_α depicted on the inverted chromosome in the schematic diagram was identified by cloning experiments (data not shown). The orientation of the immunoglobulin and T-cell receptor loci in this schema is based on the cloning experiments presented here (see text). Hatched region, sequences from band q32.3; open region, sequences from band q11.2.

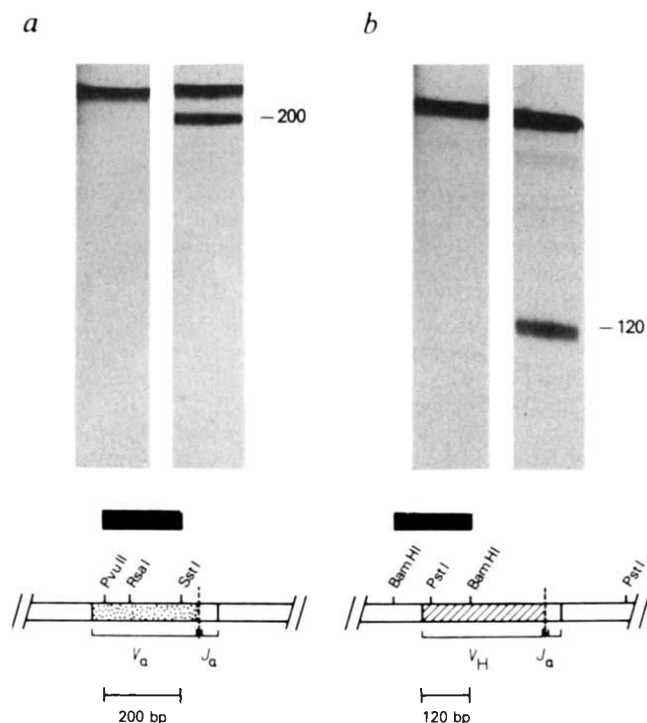


Fig. 4 S_1 nuclease analysis of SUP-T1 RNA. *a*, Transcription of a TCR V_α gene. An S_1 probe (solid bar) was prepared from 200 bp of internal TCR V_α coding sequence and 40 bp of M13 primer. Left-hand lane, control (no RNA); right-hand lane, SUP-T1 poly(A)⁺ RNA. When annealed to poly(A)⁺ RNA, all 200 bases of TCR V_α coding region (stippled region in the schematic diagram) were protected from S_1 nuclease digestion. *b*, Transcription of an immunoglobulin V_H gene. An S_1 probe (solid bar) was prepared from 270 bp of Ig V_H coding and 5' intervening sequence. Left lane, control (no RNA); right lane, SUP-T1 poly(A)⁺ RNA. When annealed to poly(A)⁺ RNA, the 120 bases of the Ig V_H coding sequence were protected from S_1 nuclease. The hatched region in the schematic diagram is the entire Ig V_H coding sequence.

The Ig V_H -TCR J_α recombinant described here clearly demonstrates that one recombination system²³⁻²⁶ can recognize both immunoglobulin²⁷ and T-cell²⁸⁻³¹ substrates and unify them; it also requires the cell to have been in a developmental state where these two distinct and disparate loci were both susceptible to a recombination event. The spacing of the signal sequences 5' of the TCR J_α and 3' of the Ig V_H suggests that the joining event would not require interposition of a D (diversity) segment if previously postulated rules of joining¹⁷ pertained. Current hypotheses suggest that these recombinases may also mediate recombination between immunoglobulin loci and other more divergent genes and signal sequences, possibly resulting in the t(14; 18)^{32,33} or t(11; 14)³⁴ translocations seen in subgroups of B-cell tumours.

The chromosome 14 inversion has been observed only in frankly malignant clones of T cells^{7,8,10}, including four out of five recently described patients with T-cell chronic lymphocytic leukaemia⁸, or in 'normal' T cells from patients with diseases that predispose to the development of leukaemia or lymphoma, such as ataxia telangiectasia³⁵⁻³⁷. Although this karyotypic finding may only reflect the aberrant joining of two distinct loci by a common recombination system at a particular point in lymphocyte development, it is also possible that this chromosomal alteration presages or contributes to malignant transformation of T cells. This might occur by activation of an as yet unidentified oncogene, or perhaps by the formation of a hybrid immunoglobulin-TCR cell-surface receptor providing an inappropriate mitogenic stimulus.

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- Yunis, J. J. *Science* **221**, 227-236 (1983).
- Klein, G. *Nature* **294**, 313-318 (1981).
- Yunis, J. J. & Soreng, A. L. *Science* **226**, 1199-1204 (1984).
- Chaganti, R. S. K. *Blood* **62**, 515-524 (1983).
- Kirsch, I. R., Brown, J. A., Lawrence, J., Korsmeyer, S. J. & Morton, C. C. *Cancer Genet. Cytogenet.* **18**, 159-172 (1985).
- Kirsch, I. R. *et al.* in *Genes and Cancer*, 583-587 (Liss, New York, 1984).
- Zech, L., Hammarstrom, L. & Smith, C. I. E. *Int. J. Cancer* **32**, 431 (1981).
- Zech, L. *et al.* *Nature* **308**, 858-860 (1984).
- Hecht, F., Morgan, R., Gemmill, R. M., Hecht, B. K. M. & Smith, S. D. *New Engl. J. Med.* **313**, 758-759 (1985).
- Hecht, F., Morgan, R., Hecht, B. K. M. & Smith, S. D. *Science* **226**, 1445-1447 (1984).
- Croce, C. M. *et al.* *Science* **227**, 1044-1047 (1985).
- Caccia, N. *et al.* *J. exp. Med.* **161**, 1255-1260 (1985).
- Kirsch, I. R., Morton, C. C., Nakahara, K. & Leder, P. *Science* **216**, 301-303 (1982).
- Yoshikai, Y. *et al.* *Nature* **316**, 837-840 (1985).
- Erikson, J., Williams, D. L., Finan, J., Nowell, P. C. & Croce, C. M. *Science* **229**, 784-786 (1985).
- Lewis, W. H., Michalopoulos, E. E., Williams, D. L., Minden, M. D. & Mak, T. W. *Nature* **317**, 544-546 (1985).
- Leder, P. *Scient. Am.* **246** (5), 102-115 (1982).
- Kabat, E. A., Wu, T. T., Bilofsky, H., Reid-Miller, M. & Perry, H. in *Sequences of Proteins of Immunological Interest*, 109-110 (US Department of Health and Human Services, Washington, DC, 1983).
- Yanagi, Y., Chan, A., Chin, B., Minden, M. & Mak, T. W. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3430-3434 (1985).
- McBride, O. W. *et al.* *Nucleic Acids Res.* **10**, 8155-8170 (1982).
- Erikson, J., Finan, J., Nowell, P. C. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5611-5615 (1982).
- Berk, A. & Sharp, P. *Cell* **12**, 721-732 (1977).
- Max, E. E., Seidman, J. G. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3450-3454 (1979).
- Sakano, H., Huppi, K., Heinrich, G. & Tonegawa, S. *Nature* **280**, 288-294 (1979).
- Sakano, H., Maki, R., Kurosawa, Y., Roder, W. & Tonegawa, S. *Nature* **286**, 676-683 (1980).
- Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. *Cell* **19**, 981-992 (1980).
- Sakano, H., Kurosawa, Y., Weigert, M. & Tonegawa, S. *Nature* **290**, 562-565 (1981).
- Hayday, A. C. *et al.* *Nature* **316**, 828-832 (1985).
- Winoto, A., Mjolsness, S. & Hood, L. *Nature* **316**, 832-836 (1985).
- Chien, Y., Gascoigne, N. R. J., Kavalier, J., Lee, N. E. & Davis, M. M. *Nature* **309**, 322-326 (1984).
- Gascoigne, N. R. J., Chien, Y., Becker, D. M., Kavalier, J. & Davis, M. M. *Nature* **310**, 387-391 (1984).
- Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C. & Croce, C. M. *Science* **226**, 1097-1099 (1984).
- Bakhsh, A. *et al.* *Cell* **41**, 899-906 (1985).
- Tsujimoto, Y. *et al.* *Nature* **315**, 340-343 (1985).
- McCaw, B. K., Hecht, F., Harnden, D. G. & Teplitz, R. L. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2071-2075 (1975).
- Kohn, P. H., Whang-Peng, J. & Levis, W. R. *Cancer Genet. Cytogenet.* **6**, 289-302 (1982).
- Taylor, A. M. R. in *Ataxia-telangiectasia. A Cellular Link Between Cancer Neuropathy and Immunodeficiency* (eds Bridges, B. A. & Harnden, D. G.) 53-81 (Wiley, New York, 1982).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).

Structure of an adenine·cytosine base pair in DNA and its implications for mismatch repair

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Mutational pathways rely on introducing changes in the DNA double helix. This may be achieved by the incorporation of a noncomplementary base on replication or during genetic recombination^{1,2}, leading to substitution mutation. *In vivo* studies³⁻⁷ have shown that most combinations of base-pair mismatches can be accommodated in the DNA double helix, albeit with varying efficiencies. Fidelity of replication requires the recognition and excision of mismatched bases by proofreading enzymes and post-replicative mismatch repair systems. Rates of excision vary with the type of mismatch and there is some evidence that these are influenced by the nature of the neighbouring sequences^{8,9}. However, there is little experimental information about the molecular structure of mismatches and their effect on the DNA double helix. We have recently determined the crystal structures of several DNA fragments with guanine·thymine and adenine·guanine mismatches in a full turn of a B-DNA helix and now report the nature of the base pairing between adenine and cytosine in an isomorphous fragment. The base pair found in the present study is novel and we believe has not previously been demonstrated. Our results suggest that the enzymatic recognition of mismatches is likely to occur at the level of the base pairs and that the efficiency of repair can be correlated with structural features.

Different theoretical models have been proposed for hydrogen bonding between cytosine and adenine, involving major^{10,11} or minor^{12,13} tautomer forms of the bases (Fig. 1*b-f*). The only experimental information to date, obtained from an NMR study¹⁴, while eliminating some of the above models, was inconclusive.

The dodecamer d(C-G-C-A-A-A-T-T-C-G-C-G) was synthesized, purified and crystallized as the ammonium salt by methods reported for two dodecamers containing G·T and A·G mismatches^{15,16}. A single crystal (0.5 × 0.2 × 0.1 mm), grown at 4 °C, was used for all diffraction experiments. The crystal data ($a = 25.37$, $b = 41.44$, $c = 65.20$ Å, space group $P2_12_12_1$, $Z = 8$) indicated isomorphism with the native dodecamer d(C-G-C-G-A-A-T-T-C-G-C-G) (ref. 17) and the G·T and A·G dodecamers^{15,16}. Two strands of the dodecamer, or one double helix, constitute the asymmetric unit of the crystal. The unit cell contains about 50% by weight of DNA (assumed density 1.5 g ml⁻¹)^{15,16}, water molecules and counterions. The X-ray intensities to 2.5 Å were measured on a diffractometer at 4 °C and after correction and averaging yielded 2,452 unique reflections, which were used for the analysis.

The starting model was a right-handed B-DNA dodecamer with atomic coordinates from the native dodecamer (ref. 17 and E. Westhof and T. Prange, personal communication). The two strands were identified by labelling them from G1 to C12 on strand 1 and G13 to C24 on strand 2. The model was gradually refined against the experimental data as a rigid body to a resolution of 3.0 Å. The bases at positions A4, C21, C9 and A16, corresponding to the sites of the mismatches, were given low occupancy. Their contribution to the calculations was effectively zero, and thus did not bias the refinement towards any particular model for the base-pair mismatch.

Refinement was continued with the Konnert Hendrickson program^{18,19}. No restraints were placed on the sugar-phosphate

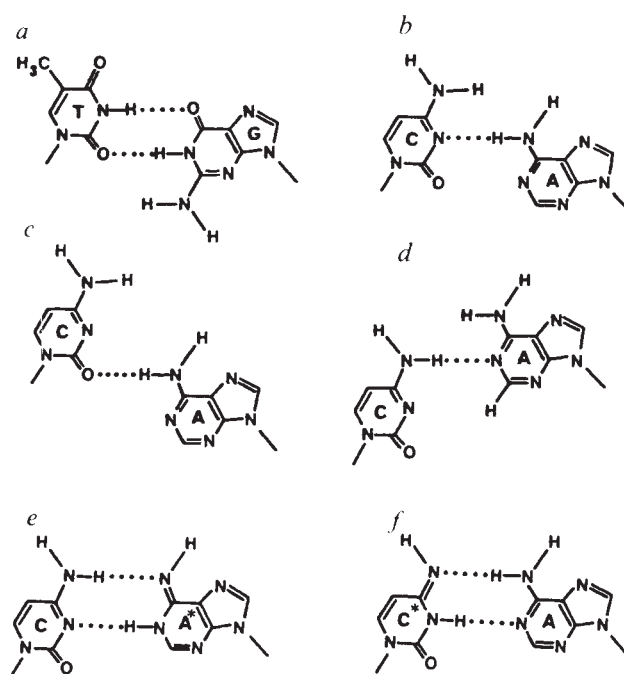


Fig. 1 Hydrogen-bonded pairs formed by noncomplementary nucleosides, similar to Watson-Crick pairs. *a*, The G·T 'wobble' base pair first proposed by Crick¹¹; *b-d*, proposed schemes for C·A pair involving major tautomer forms of bases; *e, f*, base pairs with one of the bases in a rare tautomer form (marked with an asterisk) postulated by Watson and Crick¹¹ and by Topal and Fresco¹³ on theoretical grounds to account for observed rates of mutation. These base pairs have not been observed experimentally.

torsion angles, and weak restraints were applied to the hydrogen bonds between the 10 Watson-Crick base pairs. Low occupancy factors were retained at the mismatch positions and the bases were allowed to move freely relative to each other. The mismatched bases were located and positioned in the appropriate electron density of $2F_o - F_c$ and $F_o - F_c$ maps using a graphics system (where F_o and F_c are the observed and calculator structure factors), and subsequently given full occupancy. Solvent molecules, treated as water oxygens, were located and gradually included in the calculations. No counterions could be identified with any certainty. The refinement was monitored by inspection of direct and of 'fragment' electron-density maps. The calculations converged after the location of 82 water molecules. The reliability factor R was 0.19 (the weighted reliability factor, $R_w = 0.20$) for the 2,029 reflections with intensity $I > 2\sigma$ in the range 8.0–2.5 Å.

Figure 2 shows the A·C base pair observed in the crystal structure. There are two close approaches between the bases; one of length 2.8 Å is between the N⁶ amino group of adenine and the N³ atom of cytosine and is the hydrogen bond postulated in various schemes shown in Fig. 1; the second close distance of 2.75 Å, found between the two electron acceptor groups N¹ of adenine and O² of cytosine, was unexpected. There are no solvent molecules of counterions coordinated to these groups which might thus hold them in close proximity. We conclude that this distance is indicative of a hydrogen bond. Base pairs in all DNA helices so far investigated have at least two cyclic inter-base hydrogen bonds. The formation, in such an environment, of a second hydrogen bond stabilizing the A·C base pair is entirely feasible.

Three different schemes (Fig. 2*b-d*) could account for the origin of this second hydrogen bond. The most likely is the protonation of N¹ of adenine, and the subsequent base pairing shown in Fig. 2*b*. This base pair could of course readily lose a proton to give the structures shown in Fig. 2*c* and *d*.

The pK_a (association constant) value of adenine N¹ is about

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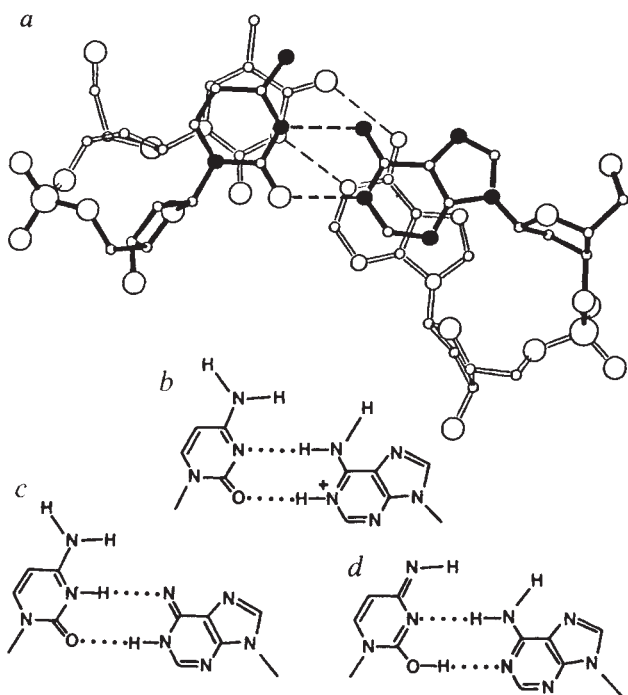


Fig. 2 *a*, The C·A base pair established by the X-ray analysis and proposed as a model for the C·A mismatch in double-helical DNA. Only one base pair, C21·A4, is shown. The second base pair, C9·A16, is identical with dimensions differing by less than 0.2 Å. The mismatched base pairs are reminiscent of the G·T wobble base pair^{15,25}, with the purines displaced laterally into the minor and the pyrimidines into the major groove. Broken lines indicate the hydrogen bonds. *b*, Schematic representation of the base pair shown in *a*. Both bases are in the major tautomeric forms and the adenine is protonated. The positive charge shown on N¹ is probably delocalized. *c*, *d*, Alternative hydrogen-bonding schemes for the C·A base pair depicted in *a* involving rare tautomeric forms of the bases. Although the contribution of these tautomers to the observed base pair cannot be ruled out, this is unlikely to be significant in view of the calculated high energy of tautomerization^{23,34}.

4.0 (ref. 20). However, the pK_a values of a base in a base pair might differ significantly from that of the constituent free base. At the pH of crystallization (7.4), the stabilization resulting from the linking of the bases by the first hydrogen bond might well be coupled with changes in the pK_a of the bases involved. Adenine may thus be protonated at N¹ with the consequent formation of the second hydrogen bond of the A·C base pair. Such closed circular hydrogen bonds are known to confer additional stabilization²⁰. There is evidence for protonated forms of adenine from high-resolution crystal structures²¹ and double-stranded poly(A) at acidic pH (ref. 22). We therefore propose the structure illustrated in Fig. 2*a* and *b* as the model for the C·A mismatch in double-helical DNA. The structure is consistent with the NMR spectra of a related dodecamer¹⁴ and with theoretical calculations²³.

Figure 3 shows a stereo view of the double helix. Although there are local fluctuations, the global conformation is essentially unaffected by the presence of the mismatched bases. The twist (35.7°), rise per base pair (3.57 Å) and number of base pairs per turn (10.1) are characteristics of B-DNA²⁴. The propeller twist (14.4°) and the backbone torsion angles are close to those reported for the native dodecamer¹⁷.

The present study and the recent analyses of seven crystal structures containing the G·T mismatch in A (refs 24, 26), B (ref. 15) and Z (refs 27, 28) helices and the G·A(*syn*) mismatch in B-DNA¹⁶, establish unequivocally that these mismatched base pairs can be accommodated in all types of DNA helices without any marked changes in the global conformations. The sugar-phosphate backbone appears to be sufficiently flexible to allow

for substantial displacement of the bases relative to the standard Watson-Crick positions, while preserving the characteristics of the particular global conformations. Base stacking is a major stabilizing force for DNA helices. As illustrated in Fig. 2, the mismatched C21·A4 base pair stacks well on the T20·A5 pair in the 3' direction. Indeed, on the 5' side of the mismatch (not illustrated) there appears to be some improvement in base stacking. Such improvements have been observed in other base-pair mismatch crystal structures¹⁵.

The solvent molecules located were mostly hydrogen bonded directly to the DNA. In the vicinity of the two mismatched bases, a water molecule links the N⁴ group of cytosine and the N⁶ of adenine and confers further stability to the C·A base pair. Similar bridging water molecules were observed for G·T base pairs²⁶⁻²⁸. In the minor groove, one water molecule was found hydrogen bonded to N³ of A4. At the other mismatch the same site is protected by the O^{3'} atom of a symmetry-related molecule. Full details of the X-ray analysis will be published elsewhere.

The probability of incorporating an incorrect base during replication and its subsequent detection and excision by polymerase is thought to be related to the thermodynamic stability of the particular base-pair mismatch in the double helix. Solution studies indicate that helices containing mismatches are less stable than those with Watson-Crick pairs only. The degree of destabilization varies with the nature of the mismatch and the base-sequence environment. Comparison by NMR of the helix-coil mid-transition point of a dodecamer indicates that G·T or A·G destabilize the double helix by 17–20 °C and A·C by 30 °C relative to the native dodecamer^{29,30}. Similar relative rates are given by melting curves for a series of nonanucleotides³¹. Recent equilibrium experiments³² with heptanucleotides indicate that at temperatures substantially below melting, the double helix is not significantly destabilized by one G·T or A·C mismatch.

Base-pair mismatches typically occur *in vivo* as rare errors in a long stretch of DNA and it is unlikely that a single A·C mismatch would destabilize the helix significantly. Enzymatic recognition at this stage would need to depend primarily on structural features. In the present crystal structure, which contains two mismatches in slightly more than one full turn, a stable B-DNA helix is observed at 4 °C. In all base-pair mismatch crystal structures analysed to date, the factors influencing helix stabilization, such as the numbers of hydrogen bonds formed by the mismatched bases (including bonding to solvent molecules) and base stacking, are comparable to those found for native structures. As in the case of proteins, the tertiary structure determined in a highly hydrated DNA crystal might indeed be the correct model for the stable *in vivo* structure. Solution studies of helix stability, on the other hand, are likely to be more relevant to the dynamic situation at the first stage of DNA replication, when an incoming base is matched to the template, and to the mechanism of proofreading by polymerase, which involves a short transient helix at the growing end of the replication fork.

We have shown from the X-ray analyses that the global conformations of DNA helices with mismatched base pairs are essentially identical to those of normal double helices. Differences are limited to the actual base pairs and involve both the structural invariants of the mismatched base pairs and the disposition of the functional groups likely to interact with repair enzymes. The characteristic feature of a Watson-Crick base pair is the pseudo-2-fold axis between the bases and the symmetrical disposition of the glycosyl bonds with respect to the vector between the sugar C1'..C1' atoms (Fig. 4). It has been suggested^{15,25,33} that this symmetry is important in recognizing a Watson-Crick pair and that further discrimination can be provided by directed hydrogen bonds interacting with functional groups of the bases in the major and minor grooves.

Evidence is now accumulating from both *in vivo* and *in vitro* studies that different base-pair mismatches are not only inserted

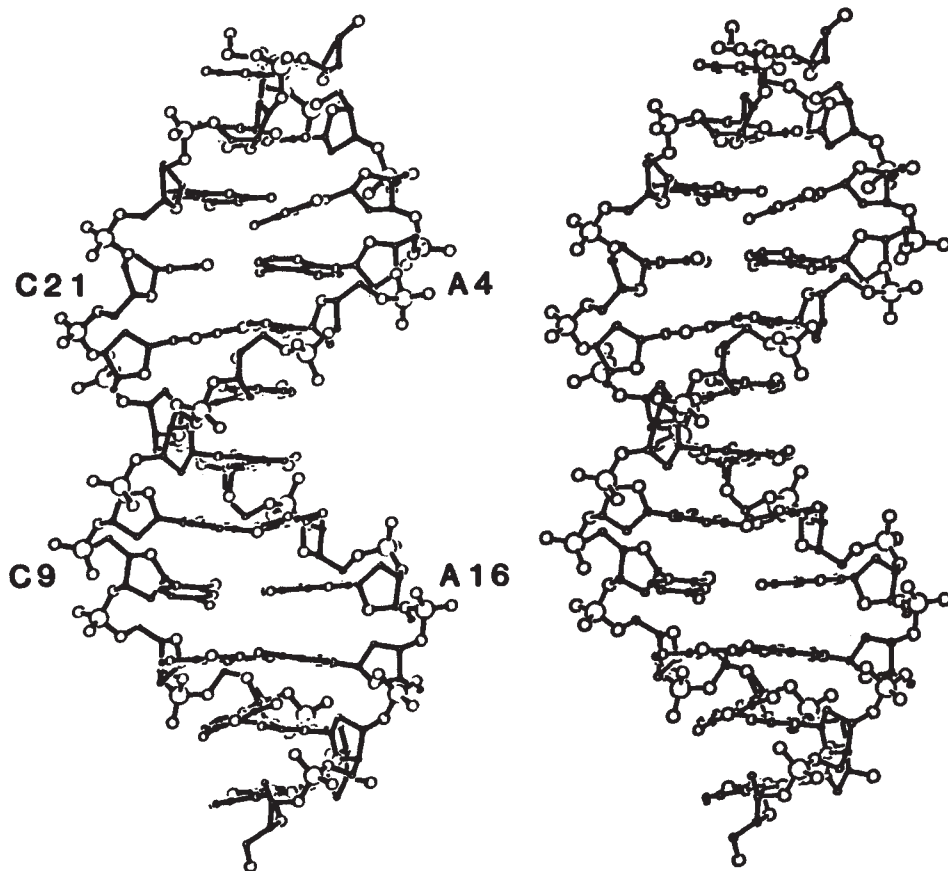
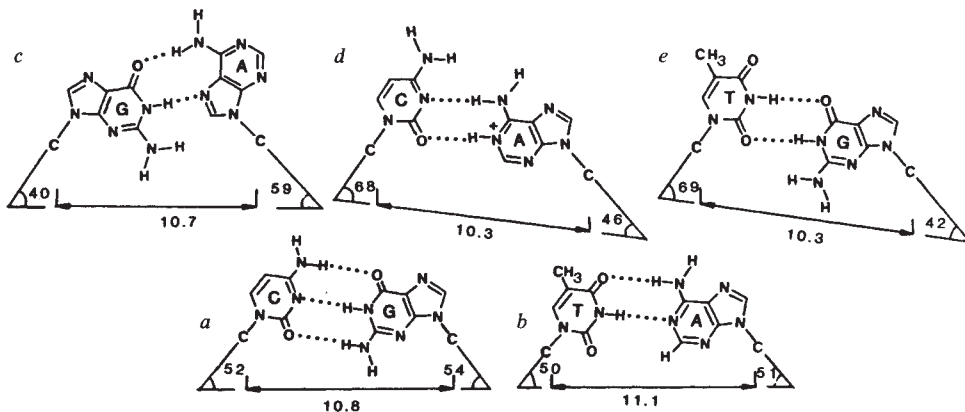


Fig. 4 Comparison of some structural features of different base pairs and correlations with efficiencies of repair. Horizontal lines indicate the separations of the $C1'$ atoms, with distances denoted in Å. The angle λ is defined as the angle between the $C1' \dots C1'$ vector and the glycosyl bond $C1'-N^1$ (N^9). Note that there are no significant differences between the $C1' \dots C1'$ separations for any of the base pairs since the double helix is able to accommodate the mismatched base pairs without substantial distortions. All numerical values are from X-ray structure determinations. *a*, *b*, Watson-Crick base pairs A·T and G·C. The base pairs have a pseudo-2-fold symmetry with the glycosyl bonds symmetrically disposed about the $C1' \dots C1'$ vector. The λ values³³ are in a narrow range of 52° – 56° . *c*, The G·A(*syn*) pair. This mismatch, which is repaired least readily, mimics a Watson-Crick pair closely, with λ values¹⁶ within $\pm 10^\circ$. *d*, A·C pair. The structure of this mismatch is intermediate between G·T and G·A(*syn*), with values (present work) -10° and $+20^\circ$ of the normal. *e*, The G·T pair. This mismatch is corrected most readily and is most asymmetrical (ref. 15 and our unpublished data).



but are also repaired with different efficiencies. *In vitro* experiments with DNA polymerases I and III indicate that G·T is proofread most efficiently, A·C with intermediate efficiency and G·A with the lowest efficiency in the ratios 15:5:1 (ref. 8, Table 5). Similar trends were found for post-replicative mismatch repair in *Escherichia coli*³⁻⁵ and in other organisms^{6,7}. As indicated in Fig. 4, there appears to be a remarkable and not previously anticipated correlation between the relative efficiencies of repair and the degree of asymmetry of the mismatched base pairs compared with Watson-Crick base pairs. It will be interesting to see whether the λ values of other base-pair mismatches (see Fig. 4 legend) can also be correlated in this fashion.

Further recognition features are provided by the disposition of the functional groups of the mismatched bases. We have previously suggested^{15,25} that, for the G·T wobble-pair, movement of the O^4 and 5-methyl groups of thymine into the major

groove might result in steric hindrance at the active site of the polymerase or the repair enzyme. A similar feature is observed in the A·C mismatch for the N^4 amino group of cytidine. It may be significant that in both the G·T and A·C structures, we found well-defined water molecules bridging the bases in the major groove. The G·A(*syn*) pair presents a very different steric disposition of the functional groups both in the major groove and in the minor groove, where the carboxyl O^2 group characteristic of all purine-pyrimidine base pairs is absent. There thus seems to be an adequate number of structural features for the recognition and differential correction of base-pair mismatches at the level of the base pairs.

The present study does not rule out the formation of A·C base pairs where one or other of the bases is in a rare tautomeric form (Fig. 1). It does not seem necessary, however, to invoke this hypothesis either for transition mutations involving A·C or

Fig. 3 Stereo view of the double helix derived from the X-ray analysis. The positions of the mismatched A·C base pairs are marked by the labelled phosphorus atoms. There are fluctuations in the local helical parameters, such as the propeller twists of the individual base pairs and the inclination of the bases to the helix axis, which, however, affect all base pairs. Local departures from average helical parameters have been observed in all single-crystal X-ray analyses of deoxyoligonucleotides published to date and have been correlated with base sequence^{35,36} and with equipartitioning of energy along the double helix³⁷.

G·T base pairs or for transversion mutations involving G·A pairs, since in all three cases base pairs in the major tautomer forms correlate adequately with the currently available information on such mutations.

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1. Radding, C. M. *A. Rev. Biochem.* **47**, 847-880 (1978).
2. Loeb, A. L. & Kunkel, T. A. *A. Rev. Biochem.* **51**, 429-457 (1982).
3. Lu, A. L., Welsh, K., Clark, S., Su, S. S. & Moldrich, P. *Cold Spring Harb. Symp. quant. Biol.* **49**, 589-596 (1984).
4. Kramer, B., Kramer, W. & Fritz, H. J. *Cell* **38**, 879-887 (1985).
5. Dohet, C., Wagner, R. & Radman, M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 503-505 (1985).
6. Claverys, S. J. P., Meján, V., Gasc, A. M. & Siccard, A. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5956-5960 (1983).
7. White, J. H., Lusnak, K. & Fogel, S. *Nature* **315**, 350-352 (1985).
8. Fersht, A. R., Knill-Jones, J. W. & Tsui, W.-C. *J. molec. Biol.* **156**, 37-51 (1982).
9. Topal, M. D., DiGiuseppi, S. R. & Sinhai, N. K. *J. biol. Chem.* **255**, 11717-11724 (1980).
10. Crick, F. H. C. *J. molec. Biol.* **19**, 548-555 (1966).
11. Watson, J. D. & Crick, F. H. C. *Nature* **171**, 694-967 (1953).
12. Drake, J. W. *Molecular Basis of Mutations* (Holden-Day, San Francisco, 1970).
13. Topal, M. D. & Fresco, J. R. *Nature* **265**, 285-189 (1976).
14. Patel, D. J., Kozlowski, S. A., Ikuta, S. & Itakura, K. *Biochemistry* **23**, 3218-3226 (1984).
15. Kennard, O. *J. biomolec. Struct. Dyn.* **3**, 205-225 (1985).
16. Brown, T., Hunter, W. N., Kneale, G. & Kennard, O. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
17. Wing, R. *et al. Nature* **287**, 755-758 (1980).
18. Hendrickson, W. A. & Konnert, J. H. in *Biomolecular Structure, Conformation, Function and Evolution* Vol. 1 (ed. Srinivasan, R.) 43-57 (Pergamon, Oxford, 1981).
19. Westhof, E., Dumas, P. & Moras, D. *J. molec. Biol.* **184**, 119-145 (1985).
20. Saenger, W. *Principles of Nucleic Acid Structure* (Springer, New York, 1984).
21. Taylor, R. & Kennard, O. *J. molec. Struct.* **78**, 1028 (1982).
22. Rich, A., Davies, D. R., Crick, F. H. C. & Watson, J. D. *J. molec. Biol.* **3**, 71-86 (1961).
23. Churprina, V. P. & Poltev, V. I. *Nucleic Acids Res.* **13**, 141-152 (1985).
24. Arnott, S. & Hukins, D. W. I. *Biochem. biophys. Res. Commun.* **47**, 1504-1509 (1972).
25. Brown, T., Kennard, O., Kneale, G. & Rabinovich, D. *Nature* **315**, 604-606 (1985).
26. Kneale, G., Brown, T., Kennard, O. & Rabinovich, D. *J. molec. Biol.* **186**, 805-814 (1985).
27. Brown, T., Kneale, G., Hunter, W. N. & Kennard, O. *Nucleic Acids Res.* (in the press).
28. Ho, P. S. *et al. EMBO J.* **4**, 3617-3623 (1985).
29. Tibanyenda, N. *et al. Eur. J. Biochem.* **139**, 19-25 (1983).
30. Patel, D. J., Koslowski, S. A., Ikuta, S. & Itakura, K. *Fedn Proc.* **43**, 2663-2670 (1984).
31. Aboul-ela, F., Koh, D., Tinoco, F. Jr & Martin, F. H. *Nucleic Acids Res.* **13**, 4811-4824 (1985).
32. Salisbury, S. & Anand, N. N. *JCS chem. Commun.* 985-986 (1985).
33. Seeman, N. C., Rosenberg, J. M. & Rich, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 804-808 (1976).
34. Rein, R., Shibata, M., Gardino-Juarez, R. & Keiber-Emmons, T. *Structure and Dynamics of Nucleic Acids and Proteins* (eds Clementi, E. & Sarma, R.) 269-288 (Adenine, New York, 1983).
35. Dickerson, R. E. *J. molec. Biol.* **153**, 410-441 (1983).
36. Shakked, Z. & Kennard, O. in *Biological Macromolecules and Assemblies* Vol. 2 (eds McPherson, A. & Jurnak, F.) 1-36 (Wiley, New York, 1985).
37. Haran, T. E., Berkovitch-Yellin, Z. & Shakked, Z. *J. biomolec. Struct. Dyn.* **2**, 397-412 (1984).

Crystallographic analysis of mutant human haemoglobins made in *Escherichia coli*

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The expression of β -globin in *Escherichia coli* has enabled us to study the functional role of individual amino-acid residues in haemoglobin (Hb) by site-directed mutagenesis¹. In contrast to mammalian Hbs, some teleost fish haemoglobins show a drastic lowering of oxygen affinity and cooperativity at low pH, a phenomenon known as the Root effect². We have produced the two mutant haemoglobins Hb Nymphéas [Cys(F9)93 β $\rightarrow$ Ser] and Hb Daphne [His(H21)143 β $\rightarrow$ Arg, Cys(F9)93 β $\rightarrow$ Ser] to investigate this allosteric property. Although these substitutions were thought to be responsible for the Root effect³, Hb Nymphéas and Hb Daphne show an increased oxygen affinity and a reduced effect of pH on oxygen affinity¹. Our X-ray crystallographic studies show that the hydroxyl group of Ser 93 β forms a hydrogen bond with Asp 94 β which is in equilibrium with the salt bridge between Asp 94 β and His 146 β . The oxygen-binding properties of Hbs Nymphéas and Daphne are accounted for by the partial disruption of the salt bridge.

The oxygen affinity of vertebrate haemoglobins is lowered by protons^{4,5}, a property known as the Bohr effect, which arises from an equilibrium between two alternative quaternary structures, the deoxy (T) structure, with low affinity for oxygen and high affinity for protons, and the oxy (R) structure, with reversed affinities⁶. In the case of mammalian haemoglobins, the transition from the T to the R state takes place even at low pH, and cooperativity remains high⁷. In some teleost fish haemoglobins such as trout IV and carp Hbs, the T structure is so favoured at low pH that the molecule remains in this state even after full oxygenation, and cooperativity is abolished^{8,9}. The more pronounced effect of protons in these fish haemoglobins, the Root effect, is caused by the extra stabilization of the T structure.

The homology in the amino-acid sequence between human and carp Hbs is of the order of 50% and it is not clear which residues are responsible for the Root effect. Using model building, Perutz and Brunori³ reasoned that the salt bridge between the C-terminal His 146 β and Asp or Glu 94 β which stabilizes

the T structure would be strengthened in fish Hbs, because Ser at position 93 β would form hydrogen bonds with the carbonyl and amide groups of His 146 β . The additional stabilization of the C-terminal salt bridge would favour the T structure, particularly at low pH when the imidazole of His 146 β is ionized, and would give rise to the Root effect. We have developed an experimental method to test this hypothesis.

Functionally active Hb has been reconstituted from β -globin produced in *E. coli*, allowing any mutant to be prepared by site-directed mutagenesis. Nagai *et al.*¹ have recently produced the two mutants Hb Nymphéas [Cys(F9)93 β $\rightarrow$ Ser] and Hb Daphne [Cys(F9)93 β $\rightarrow$ Ser, His(H21)143 β $\rightarrow$ Arg] to investigate the effect of the Cys 93 β $\rightarrow$ Ser and the additional His 143 β $\rightarrow$ Arg substitutions in fish Hbs. Hb Nymphéas showed a slightly increased oxygen affinity and reduced alkaline Bohr effect¹. The oxygen affinity of Hb Daphne was markedly increased and the Bohr effect reduced¹. We decided to determine their structures by X-ray analyses in order to investigate why the Cys 93 β $\rightarrow$ Ser substitution did not produce the Root effect.

Hbs Nymphéas and Daphne were crystallized in the T state¹⁰ and their structures were determined by difference Fourier syntheses to 2.8 Å and 2.45 Å resolution, respectively. In the difference map for Hb Nymphéas, a negative peak due to the sulphur to oxygen replacement is centred at Cys 93 β and weaker pairs of positive and negative density occur in the vicinity (Fig. 1). The map also reveals that any structural changes that have occurred are close to the substitution, confirming that there is no other structural difference between the β -chain made in *E. coli* and the native human chain. The paired positive and negative density at His 146 β shows that it has become displaced. At a lower contour level, a negative feature is observed on the distant side of Asp 94 β , indicating that it has moved closer to the serine and that it forms a hydrogen bond with the hydroxyl group of Ser 93 β which is in equilibrium with the normal salt bridge between Asp 94 β and His 146 β . The partial disruption of this salt bridge between His 146 β and Asp 94 β destabilizes the T state and reduces the Bohr effect. The positive peak on the opposite side of the serine may be a diffraction effect or a newly ordered water molecule, as the hydroxyl group is a better hydrogen-bond former than the sulphhydryl group.

In addition to changes around Ser 93 β in Hb Nymphéas, the difference map of Hb Daphne shows extensive movement of side chains around the His 143 β $\rightarrow$ Arg substitution (Fig. 2). Electrostatic repulsion by the positively charged arginine side chain causes Lys 82 β to move away slightly, and this in turn

Fig. 1 A stereoscopic Fourier difference map at 2.8 Å resolution of Hb Nymphéas minus native HbA plotted on coordinates for native HbA. Positive density is indicated by solid contours, negative by broken contours. Water molecules are included.

Methods. Mutant Hb Nymphéas [Cys(F9)-93β→Ser] was produced in *E. coli* by the procedure of Nagai *et al.*¹ and crystallized in space group P2₁ under conditions described by Perutz for deoxy-HbA (ref. 10). X-ray data sets were collected using a Watts-Hilger four-circle diffractometer. Averaged cell dimensions for Hb Nymphéas (and native) crystals (*a*, *b*, *c* in Å; β angle): 63.17 (63.15), 83.39 (83.59), 53.75 (53.80), 99.34° (99.34°). A total of 26,694 reflections were collected from two Hb crystals, and the maximal intensity degradation of reference reflections was 18.4%. Agreement between the 12,870 unique set and their symmetry-related reflections was 5.5% on intensity. No overlapping scaling reflections were collected, and each crystal data set was scaled independently against the native deoxy amplitudes from the 1.74-Å deoxy-HbA structure¹¹. The residual for scaling (R_{scale}) against native deoxy-Hb was 6.6% with scale and temperature factors applied. The r.m.s. density (σ) in the difference map was $0.0182 \text{ e} \text{ Å}^{-3}$. Contour levels are set at 5σ density. No significant features were observed elsewhere in the map at $0.054 \text{ e} \text{ Å}^{-3}$ contouring, and the features observed here were also observed at the site related by the molecular diad, which is non-crystallographic. The map $[F(\text{Ser } 93\beta)_{\text{calc}} - F(\text{native})_{\text{calc}}] \exp(i\alpha_{\text{native}})$, where α_{native} is the native phase, calculated at 2.8 Å (R_{scale} 1.6%), revealed a positive density diffraction feature enveloping the negative feature at the substitution site and at 1/20th the intensity of the negative feature; it does not affect interpretation of the map presented here.

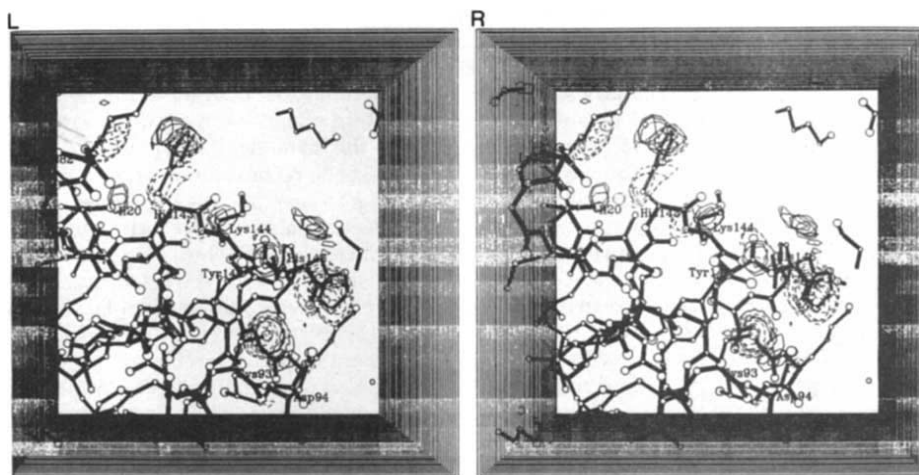
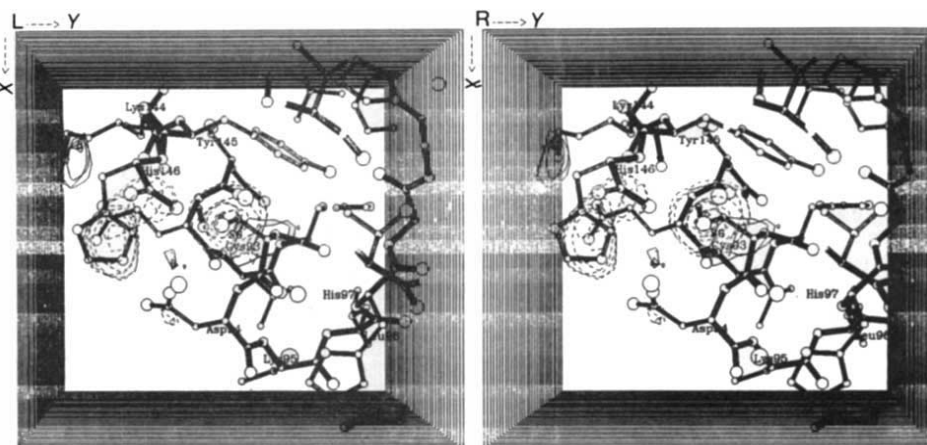


Fig. 2 A difference map at 2.45 Å of Hb Daphne [Cys(F9)93β→Cys, His(H21)-143β→Arg] minus HbA. Cell dimensions 63.23, 83.31, 53.75, β = 99.40°. Positive density is indicated by solid contours, negative by broken contours.

Methods. A total of 39,933 reflections—constituting 19,327 unique *hkl* parameters, $R = 5.2\%$ —were collected from one crystal. The maximum degradation of reference reflections was 14.6%. R scaling to native, 8.9%; σ , $0.0269 \text{ e} \text{ Å}^{-3}$. No significant features were observed elsewhere in the map, and small differences in the magnitude of the displacements were observed between the symmetry-related sites.

causes a small displacement of the corner connecting the E and F helices. The negative and positive features near Arg 143β arise from the superposition of many changes. A phosphate ion seems to have become sandwiched between Arg 143β and the neighbouring Lys 144β, which also moves away. One of the water molecules observed in the refined 1.74-Å deoxy-Hb structure¹¹ is displaced.

In teleost fish Hbs, Asp 94β is replaced by Glu which, having a longer side chain, would be able to maintain the salt bridge with His 146β and would not form a hydrogen bond with Ser 93β. Hence, an Asp 94β→Glu replacement, in addition to the Cys 93β→Ser substitution, appears to be necessary to produce the Root effect. We are presently testing this hypothesis.

A substitution of Cys by Ser is expected to cause a small perturbation in protein molecules, since these residues are similar in size and properties. The present study shows that even such a seemingly minor substitution can cause secondary changes in the structure and function of proteins. Hence, care should be taken in interpreting structural effects of mutations in the absence of X-ray crystallographic studies.

In the course of evolution, Hb molecules have undergone extensive amino-acid substitutions. However, they have retained the same three-dimensional structure as well as basic allosteric properties, such as haem-haem interaction and the Bohr effect. On the other hand, some functional divergence is observed¹²,

of which the Root effect in teleost fish Hbs^{8,9} and the bicarbonate effect in crocodile Hbs¹³ are examples. Our aim is to identify amino-acid residues which are responsible for functions specific to a particular species and to distinguish functionally important substitutions from neutral ones¹⁴; this can be done by replacing amino acids by site-directed mutagenesis, thus reversing evolution.

We thank Dr M. Perutz for his continuous encouragement, Dr G. Fermi for help with computation, Colin Young for growing cells, and Dr Peter Esnouf for the gift of factor X. This paper is dedicated to the memories of Professor H. Lehmann and Marcus Kress. This work is supported by NIH grant R01 HL 31461-01.

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1. Nagai, K., Perutz, M. F. & Poyart, C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7252-7255 (1985).
2. Root, R. W. *Biol. Bull.* **61**, 427-463 (1931).
3. Perutz, M. F. & Brunori, M. *Nature* **299**, 421-426 (1982).
4. Kilmartin, J. V. & Rossi-Bernardi, L. *Physiol. Rev.* **53**, 836-890 (1973).
5. Bohr, C., Hasselbalch, K. & Krogh, A. *Skand. Arch. Physiol.* **16**, 402-412 (1904).
6. Perutz, M. F. *Nature* **228**, 726-739 (1970).
7. Imai, K. *Allosteric Effects in Haemoglobins* (Cambridge University Press, 1982).
8. Tan, A. L., Nobel, R. W. & Gibson, Q. H. *J. biol. Chem.* **248**, 2880-2888 (1973).
9. Brunori, M. *Curr. Topics cell. Regulation* **9**, 1-39 (1975).
10. Perutz, M. F. *J. Crystal Growth* **2**, 54-56 (1968).
11. Fermi, G., Perutz, M. F., Shannon, B. & Fourme, R. *J. molec. Biol.* **175**, 159-174 (1984).
12. Perutz, M. F. *Molec. Biol. Evol.* **1**, 1-28 (1983).
13. Bauer, C. & Jelkmann, W. *Nature* **269**, 825-827 (1977).
14. Kimura, M. *Scient. Am.* **241** (5), 94-104 (1979).

Strong signals from streptavidin–biotin

from R. M. Buckland

The robust attraction between a bacterial protein and vitamin H could boost the sensitivity of immunoassays, improve DNA probes and aid in protein purifications. Its role in nature remains a mystery.

EGG white and culture supernatant from the soil bacterium *Streptomyces avidinii* may not, at first sight, appear to have much in common. Yet from these disparate sources, two unusual and related proteins have been isolated that are capturing the interest of chemists, biochemists and biologists. Avidin, from egg white¹, and streptavidin, from *Streptomyces avidinii*², are tetrameric proteins containing four high-affinity binding sites for biotin, a small water-soluble vitamin. The dissociation constant (K_D) for avidin or streptavidin–biotin interactions is approximately 10^{-15} M, a figure few other non-covalent interactions in nature can match³. By exploiting this high affinity, researchers have already improved the sensitivities of immunoassays, immunocytochemical techniques and DNA probe diagnostics, and even broader applications seem likely.

Several properties of streptavidin enable it to bind more specifically than avidin at the pH used in most assay systems⁴. Because avidin has an isoelectric point (pI) above 10, and because it is glycosylated, the protein can stick to DNA and cell membranes. In contrast, streptavidin is not glycosylated and exhibits a pI

between 5.5 and 6.5². Streptavidin is therefore the reagent of choice for immunoassay and DNA probe detection studies where nonspecific binding to lectins, phospholipids and nucleic acids can lead to high background. Purified streptavidin is commercially available from Apcel Ltd and several other biochemical companies.

Although the avidin–biotin system has been part of immunochemical tissue staining for many years⁵, its use for enhancing signal detection in immunoassays was not described until 1979⁶. Under suitable conditions, several molecules of biotin can be covalently coupled to antibody without adversely affecting the antigen binding capacity⁶. The streptavidin–biotin interaction can then link antigen–antibody complexes to suitable signal generation systems. The rapid and essentially irreversible binding of biotin to streptavidin, along with the ability to attach several biotin molecules to a single antibody molecule, makes the system particularly attractive for improving sensitivity in immunoassay diagnostics.

Each of the streptavidin–biotin assay variants illustrated in Fig. 1 offers particular advantages. In the simplest form, streptavidin covalently labelled with ¹²⁵I, fluor or enzyme, for example, is used to detect a biotinylated antibody (Fig. 1a). This variant is particularly appropriate for low molecular weight labels, although large enzyme molecules can also be attached to streptavidin, via spacer arms, without sacrificing assay performance⁷.

Double decker

Because of its multi-valency, streptavidin can also link two or more biotinylated molecules in a 'bridge' or 'sandwich' mode (Fig. 1b). In this variant, both antibody and label (usually enzyme) are biotinylated, and streptavidin then serves to join these two components. Because a single tetrameric streptavidin molecule can bind up to four molecules of biotin, this technique amplifies the signal: each streptavidin molecule bound to a biotinylated antibody can also bind three biotinylated enzyme labels.

A third variant achieves even higher amplification using three-dimensional complexes of streptavidin and biotinylated enzyme labels (Fig. 1c). Prior to use, the complexes are formed by mixing streptavidin and biotinylated enzyme label in defined stoichiometry. This

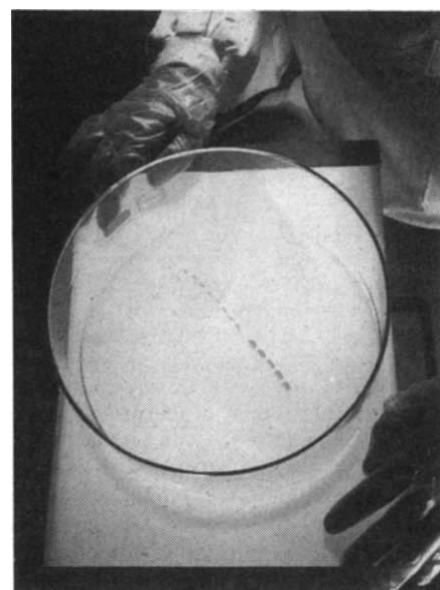


Fig. 2 Single-band purity of streptavidin on SDS-PAGE.

systems attains strong signals that maximize sensitivity and minimize signal-to-noise ratios.

Beyond diagnostics

Immunological diagnostics has embraced the streptavidin–biotin system in enzyme-linked immunoassays for carcino-embryonic antigen⁸, herpes simplex virus antigen⁹ and flavivirus antigens¹⁰. Several commercial kits that use the system have recently appeared on the market. Streptavidin's binding specificity also figures importantly in signal generation for DNA probe diagnostics. David Ward and his colleagues have described methods for the enzymatic incorporation of biotin-labelled thymidine triphosphate (TTP) analogues into DNA¹¹. These biotinylated DNA probes can hybridize normally with complementary DNA¹², providing an anchor for streptavidin and its attached signal generation system.

Coupling the signal to the probe allows diagnosis at the DNA rather than the protein level (Fig. 3). Biotinylated probes can detect as little as 100 fg of DNA on dot and Southern blot hybridizations. Several variations of the procedure have been described, including one based on photobiotin, a photo-activated analogue of biotin¹³. Plans to develop and commercialize this new technology are now under way.

The power of the streptavidin–biotin system is not limited to diagnostic assays. The system has also proved useful for

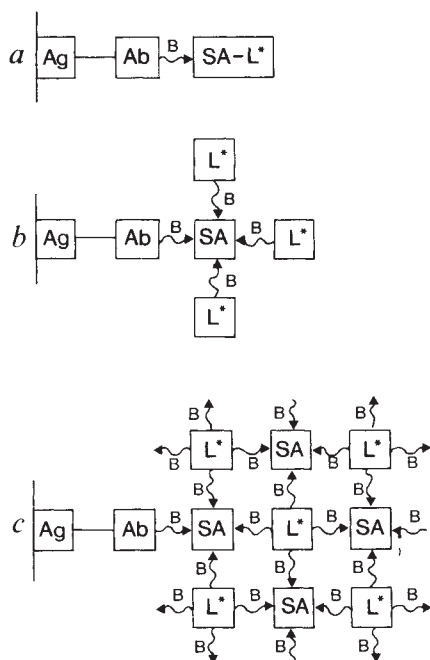


Fig. 1 Variants of the streptavidin–biotin system for antigen detection in immunoassay applications. Details of the methods are given in the text. Ag, antigen; Ab, antibody; B, biotin; SA, streptavidin; L*, label.

immunochemical protein detection in Western blots^{14,15}. Bound to biotinylated monoclonal antibodies, detergent-solubilized membrane antigens can be isolated by immunoaffinity chromatography with immobilized streptavidin¹⁶. The interaction between streptavidin and biotin-labelled human growth hormone has also helped purify lactogenic and somatogenic hormone receptors from rabbit liver and mammary membranes¹⁷, and the system's potential for isolating insulin receptors has been assessed¹⁸. Finally, the avidin-

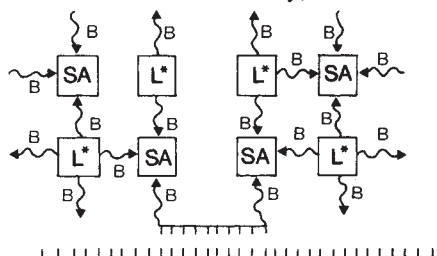


Fig. 3 Use of the streptavidin-biotin system in DNA probe diagnostics. SA, streptavidin; B, biotin; L, label. Biotin provides an anchor for the streptavidin signal generation complex.

biotin complex can facilitate fusion of myeloma cells and β -lymphocytes to form hybridomas¹⁹, and deliver avidin-attached cytotoxic drugs to tumour cell surfaces via biotinylated, tumour-specific antibodies²⁰. As interest in the unique features of the streptavidin-biotin system continues to grow, these novel applications are sure to be joined by a host of others. □

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1. Eakin, R.E., Snell, E.E. & Williams, R.J. *J. biol. Chem.* **140**, 535-543 (1941).
2. Chalet, L. & Wolf, K.J. *Archs Biochem. Biophys.* **108**, 1-5 (1964).
3. Green, N.M. *Adv. Protein Chem.* **29**, 85-133 (1975).
4. Hofmann, K., Wood, S.W., Brinton, C.C., Montibeller, J.A. & Finn, F.M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4666-4668 (1980).
5. Bayer, E.A., Wilchek, M. & Skutelsky, S. *FEBS Lett.* **68**, 240-244 (1976).
6. Guesdon, J.L., Ternynck, T. & Avrameas, S. *J. Histochem. Cytochem.* **27**, 1131-1139 (1979).
7. Bonnard, C., Papermaster, D.S. & Kraehenbuhl, J.P. in *Immunolabelling for Electron Microscopy* (eds. Polak, J.M. & Varndell, I.M.) 95-111 (Elsevier, Amsterdam, 1984).
8. Buchegger, F., Haskell, C.M., Carrel, S., Rosenberger, M., Testuz, J. & Mach, J.P. *Protides Biol. Fluids* **31**, 1025-1030 (1984).
9. Nerurkar, L.S. *et al. J. clin. Microbiol.* **20**, 109-114 (1984).
10. Gould, E.A., Buckley, A. & Cammack, N. *J. virol. Meth.* **11**, 41-48 (1985).
11. Langer, P.R., Waldrop, A.A. & Ward, D.C. *Proc. natn. Acad. Sci. U.S.A.* **78**, 633-637 (1981).
12. Leary, J.J., Brigati, D.J. & Ward, D.C. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4045-4049 (1983).
13. Forster, A.C., McInnes, J.L., Skingle, D.C. & Symons, R.H. *Nucleic Acids Res.* **13**, 745-761 (1985).
14. Brower, M.S., Brakel, C.L. & Garry, K. *Analyt. Biochem.* **147**, 382-386 (1985).
15. Nikolau, B.J., Wurtele, E.S. & Stumpf, P.K. *Analyt. Biochem.* **149**, 448-453 (1985).
16. Updyke, T.V. & Nicholson, G.L. *J. immun. Meth.* **73**, 83-95 (1984).
17. Haeuptle, M.T., Aubert, M.L., Djiane, J. & Kraehenbuhl, J.P. *J. biol. Chem.* **258**, 305-314 (1983).
18. Finn, F.M., Titus, G. & Hofmann, K. *Biochemistry* **23**, 2554-2558 (1984).
19. Lo, M.M.S., Tsong, T.Y., Conrad, M.K., Strittmatter, S.M., Hester, L.D. & Snyder, S.H. *Nature* **310**, 792-794 (1984).
20. Philpott, G.W., Kulozcki, A., Grass, E.H. & Parker, C. *W.J. J. Immun.* **125**, 1201-1209 (1980).

The latest in Saint Louis

With over 600 exhibits and nearly 6,000 paper presentations, this year's FASEB meeting may exhaust even the most energetic of visitors.

RIGHT near the FASEB Lounge in the St Louis Convention Center, Packard Instruments will be exhibiting their cost-conscious version of the older Tri-Carb 2000CA for **liquid scintillation analysis**. Packard's new Tri-Carb 2000 is modelled in design and technique on its analysing forerunner, but uses microprocessor control rather than an IBM PC hook-up, and designates as options several features that were standard on the 2000CA (Reader Service No. 100). The advantage: a price cut of over \$8,000 (US), and the capability for a full upgrade when the wallet allows. The Tri-Carb 2000, including



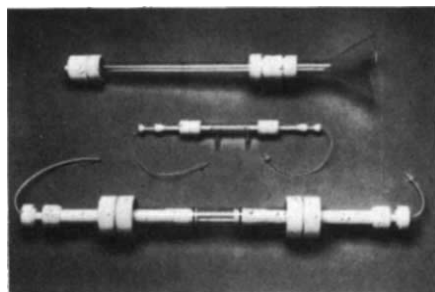
The Tri-Carb 2000 goes back to basics.

a sample capacity up to 408 large or 792 small vials, three-dimensional spectrum analysis and a linear spectrum analyser, will premier at booth J48.

Six rows west of Packard's booth, a dedicated **amino acid analysis** system from Waters will be on display (Reader Service No. 101). The Pico Tag method gives quantitative analysis of as little as 10 μ g of plasma in less than 90 minutes. Waters' method is based on a one-step precolumn derivatization with phenylthiocyanate (PITC), which yields phenylthiocarbamyl (PTC) amino acid derivatives containing a single ultraviolet-active chromophore. The success of the chemistry package (including column, reagents and protocol) can only be guaranteed, however, when used in conjunction with Waters' equipment. A complete Pico Tag analysis assembly will be in booths C43 to 49 (odd) and D42 to 48 (even).

Bio-Rex MP chromatography columns will be big at Bio-Rad's booth E43. The **medium pressure columns** were designed

with versatility in mind and are resistant to solvent and pH extremes (Reader Service



Bio-Rad columns come with fittings for HPLC pumps.

No. 102). The Bio-Rex flow adaptor allows bed height adjustment without sacrificing pressure or solvent resistance. Both 20 and 40 μ m bed supports are available on the columns, which range in diameter from 1.0 to 7.5 cm, and prices vary according to size.

Brinkmann's model 2010 particle size analyser will put in an appearance at booths D30 to D35. The analyser incorporates an image analysis microscope into the basic measuring unit, which will size particles from 0.7 μ m to 1,200 μ m in any media with its scanning laser system (Reader Service No. 103). The model 2010's base price is \$31,200 (US), but the



Brinkman's marriage of particle sizing and image analysis.

analyser can be linked to an IBM PC/XT in a \$38,000 package. Data arrive as statistics for density, distribution, volume and number. A \$7,800 (US) image analysis accessory can also be purchased to acquire sample images.

By automating the development phase and refining gel processing, Pharmacia has put together an **electrophoresis system** that will run, stain and destain IEF gels in one hour and PAGE gels in under an hour and a half. Pharmacia's Phastsystem, shown in FASEB booths L51 to L55 (odd)

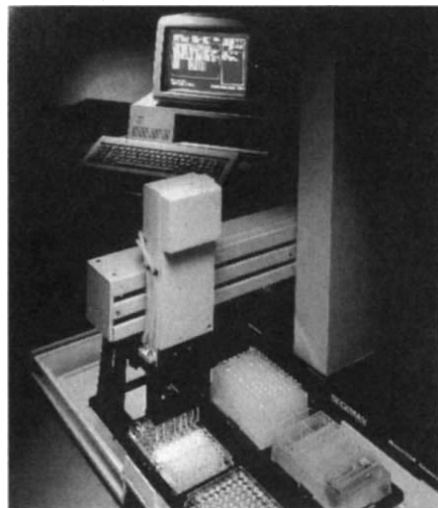


Phastsystem saves time and gel.

and M60 to M64 (even), contains the instrumentation for separation and development along with microprocessor control to standardize operating procedures (Reader Service No. 104). Inch-square gels to fit the unit come ready-made from Pharmacia. Phastsystem sells for about \$6,000 (US).

Steps in sample prep.

Across from the exhibit entrance in booths F41 and G40, Beckman Instruments will be demonstrating their new laboratory 'work-station', the Biomek 1000 (Reader Service No. 105). This **integrated robotics unit** defies traditional perceptions of robotics: instead of an arm surrounded by specialized stations, the Biomek 1000 consists of four instruments incorporated into one 28 × 24 × 17.5-inch device. In meshing different functions together, Beckman has accelerated sample processing, but restricted the instrument's flexibility. Biomek can perform pipetting, diluting, dispensing, plate washing and photometric analysis, but will not grind, weigh or carry out any tasks not frequently used in life sciences research. It is well suited to methodologies such as hybridoma screening and selection, ELISA and other immuno-

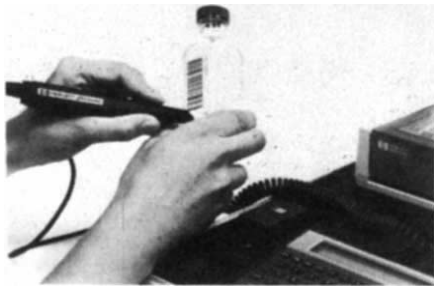


The Biomek workstation puts robotics in a new light.

assays. The system can cost between \$18,500 and \$35,000 (US), depending on the interchangeable components required for particular functions. A separate IBM PC controls Biomek with menu-driven software.

At Hamilton's booths in the southern arm of the convention centre, another **fluid processor** will be making its mark. The Microlab 2000 system can process up to 500 sample tubes with as many as 50 reagents in one run, and transfer samples, diluents, reagents and other liquids in volumes from 2 µl to 25 ml (Reader Service No. 106). Hamilton's processor is also controlled by an IBM PC, with multi-tasking software that will execute up to four programs simultaneously. The Microlab 2000, on display in booths F12 and F14, costs less than \$24,500 (US) complete with the computer and colour monitor. Applications include several types of immunoassays, serological testing and automatic sample tray loading.

As automated sample preparation becomes increasingly popular, keeping track



Bar coding sets the records straight.

of processed samples may become increasingly difficult. Last month Hewlett Packard introduced a **portable bar-code system** that may help clear up the confusion (Reader Service No. 107). The HP 7690A can both label samples with high-density ink jet printing, and identify them using a bar-code wand. With the HP-75D portable computer, the system will generate numeric or alphanumeric series on labels, encode location, date or time and retrieve previously stored information. Hewlett Packard includes an initial supply of labels, label covers and ink-jet cartridges with its \$3,000 (US) HP 7690A. HP representatives in booths A44 and A45 will have more information.

A clean sweep

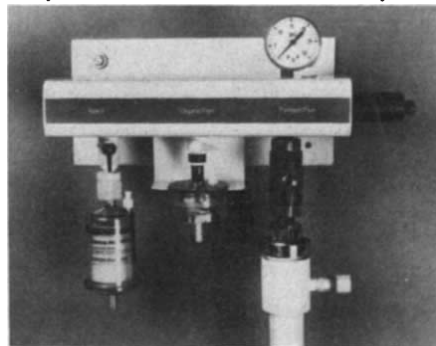
Two Sybron divisions at FASEB will be pushing their purity products in booths 144 to 48 (even). Sterilization by membrane filtration will grab the spotlight in Nalge's corner, as they feature their latest **disposable filter units** in expanded capacities, pore sizes and membrane materials (Reader Service No. 108). All Nalge's filterware has a closure design that allows storage in the separate receiver, colour-coded collars for membrane types and graduated uppers and receivers. Membrane compositions include the cellulose nitrate membrane in 0.2, 0.45 and 0.8 µm sizes, the tissue-culture-grade cellulose acetate membrane in 0.2 and 0.45 µm sizes and the nylon membrane in 0.1, 0.2



Nalge's filter units are now colour-coded.

and 0.45 µm sizes, each intended for a particular application. Most units come in 30 to 1000 ml capacities.

Next door at Barnstead, the **NANO-pure filter manifold accessory** for NANO-pure II cartridge deionizers will make its debut (Reader Service No. 109). Barnstead's new manifold enables specialized final filtration options such as ultrafilters or organic-free HPLC attachments to be connected simultaneously to the base NANOpure II assembly. Constructed of non-leaching high molecular weight polyethylene, the manifold accessory has



Many routes to purity from Barnstead.

provisions for recirculation to stave off deadlegs and recontamination. It comes with tubing, fittings, adapters and installation instructions for \$265 (US).

Chilling news

A **wide-range cryoscope** from Precision Systems can perform molecular weight determinations for a variety of known or unknown substances, with complete sample recovery. In FASEB booth K41, the Cryette WR will be on show for chemists who work with solvents such as benzene, nitrobenzene, cyclohexane, *p*-xylene, freon 112 and others (Reader Service No. 110). The instrument's versatility corresponds to its ability to measure freezing points from -6° to 26°C. Its

versatility, is also reflected in its price: \$8,600 (US). But Precision System's device measures freezing points automatically with a precision of 0.001°C, giving molecular weight determinations within 1 per cent accuracy. The Cryette accommodates 2.0 to 2.5 ml samples and supplies results within 2 minutes.

Queue Systems has a **cryogenic preservation chamber** that attains temperatures



Queue has a deep freeze without liquid nitrogen, as low as -135°C without using liquid nitrogen. A patented mechanical system in the Cryostar keeps the cold weather going more economically than liquid nitrogen systems can (Reader Service No. 111). At FASEB, the Cryostar will occupy

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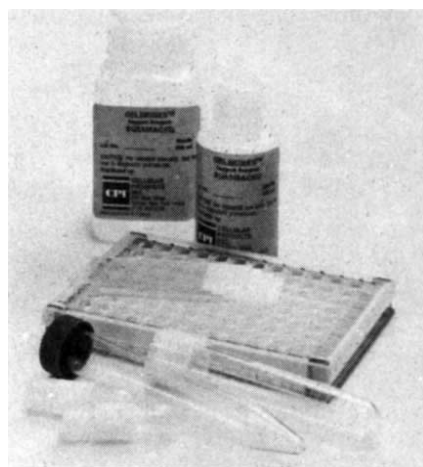
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Reader Service No.31

booth E16. The unit is available in two sizes: the model 7140, which holds over 17,000 2 ml vials, and the 7160, which accommodates over 7,000. Both operate with 100 per cent non-flammable fluorocarbon refrigerants.

Critical chemicals

A test for **AIDS antibodies and antigens** will be part of Cellular Products' exhibit in the northwest corner of the St Louis Convention Center. CPI's Retro-Tek ICA/AIDS kit is a confirmatory slide test for research laboratories, with greater than 99 per cent sensitivity (Reader Service No. 112). The immunocytological assay contains an internal negative control and specificity close to 100 per cent, but is much more economical than Western blot



Retro-Tek kits put viruses to the test.

assays. Available in immunofluorescent or immunoperoxidase formats, the \$150 (US) kits include equipment and reagents for 25 tests.

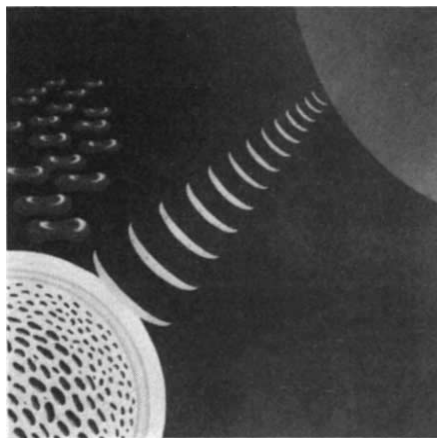
Also at CPI's booth A40, an AIDS virus capture assay will be on display. The Retro-Tek VCA/AIDS ELISA kit has greater specificity and sensitivity than reverse transcriptase enzymatic assays because it detects viral antigens (Reader Service No. 113). CPI's test provides semiquantitative results and will not cross-react with other human T-cell lymphotropic viruses. The \$175 (US) kit contains materials for 96 tests; direct absorbance readings, which indicate the progress of the disease, complete the process in about 3 hours.

At Boehringer Mannheim Biochemicals' booths N41 and N43, two **enzymes for N-glycan analysis** have surfaced where once was only one. BMB has separated the two distinct activities found in endoglycosidase F, yielding preparations with greater than 99 per cent purity (Reader Service No. 114). The two N-glycan cleaving activities, endo- β -N-acetylglucosaminidase F and peptide: N-glycosidase F have wisely been dubbed ENDO F and PNGase F by BMB. The preparations

show no detectable proteolytic or exoglycolytic activities, and cost between \$100 and \$350 (US) depending on grade and quantity.

A **protein dye for Western blots** will be amongst the chemicals exhibited in Janssen Life Sciences booths E61 to E63 at FASEB. The FerriDye kit for immunochemistry offers the rare advantage of being able to stain proteins on nylon-based membranes, as well as nitrocellulose (Reader Service No. 115). Janssen's dye contains colloidal iron particles that selectively bind to blotted SDS-denatured polypeptides with 1 ng protein per mm² sensitivity. A subsequent Perl's reaction stains the protein bands blue. Background impurities tend to crop up more frequently with FerriDye than with similar stains such as Coomassie brilliant blue or amino black, so extra care must be taken to work cleanly during preparation. A FerriDye kit consists of the reagents for about 20 standard (10 × 15 cm) blotting membrane sheets; each has a shelf life of at least six months and costs \$90 (US).

Amersham International now offers **erythropoietin** derived from recombinant DNA technology in three different product lines (Reader Service No. 116). For studies of red cell development and function, ultra-pure human recombinant erythropoietin boasts a high specific biological activity. Human recombinant TC erythropoietin has been formulated for use in tissue culture media and, although as pure as the ultra-pure product, this type has lower specific biological activity. Finally, an iodinated analogue of human recombinant erythropoietin for RIA



Erythropoietin: behind the scenes at the birth of red blood cell.

determinations and receptor studies completes the roster. Amersham's research division will be showing in booths F24 through F28 (even). □

These notes are compiled by Karen Wright from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal.